A Sphingosine 1-Phosphate 1 Receptor Agonist Modulates Brain Death–Induced Neurogenic Pulmonary Injury

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Lung transplantation remains the only viable therapy for patients with end-stage lung disease. However, the full utilization of this strategy is severely compromised by a lack of donor lung availability. The vast majority of donor lungs available for transplantation are from individuals after brain death (BD). Unfortunately, the early autonomic storm that accompanies BD often results in neurogenic pulmonary edema (NPE), producing varying degrees of lung injury or leading to primary graft dysfunction after transplantation. We demonstrated that sphingosine 1-phosphate (S1P)/analogues, which are major barrier-enhancing agents, reduce vascular permeability via the S1P1 receptor, S1PR1. Because primary lung graft dysfunction is induced by lung vascular endothelial cell barrier dysfunction, we hypothesized that the S1PR1 agonist, SEW-2871, may attenuate NPE when administered to the donor shortly after BD. Significant lung injury was observed after BD, with increases of approximately 60% in bronchoalveolar lavage (BAL) total protein, cell counts, and lung tissue wet/dry (W/D) weight ratios. In contrast, rats receiving SEW-2871 (0.1 mg/kg) 15 minutes after BD and assessed after 4 hours exhibited significant lung protection (~50% reduction, $P = 0.01$), as reflected by reduced BAL protein/albumin, cytokines, cellularity, and lung tissue wet/dry weight ratio. Microarray analysis at 4 hours revealed a global impact of both BD and SEW on lung gene expression, with a differential gene expression of enriched immune-response/inflammation pathways across all groups. Overall, SEW served to attenuate the BD-mediated up-regulation of gene expression. Two potential biomarkers, TNF and chemokine CC motif receptor-like 2, exhibited gene array dysregulation. We conclude that SEW-2871 significantly attenuates BD-induced lung injury, and may serve as a potential candidate to improve human donor availability.

Keywords: neurogenic pulmonary edema; lung injury; sphingosine 1-phosphate; sphingolipids; lung transplant donors

Over the past decade, lung transplantation has become an increasingly important mode of therapy for patients with a variety of endstage lung diseases. The vast majority of lung donors are individuals with brain death (BD). However, the early autonomic

storm accompanying brain BD triggers the development of systemic and pulmonary inflammatory responses, which lead to increased pulmonary endothelial permeability (1, 2) and to the sympathetic vasoconstriction of the systemic and pulmonary vasculature. These changes, in addition to possible ischemia-reperfusion injury, disrupt the integrity of the alveolar capillary membrane, resulting in neurogenic pulmonary edema (NPE) (1–4). Increased pulmonary interstitial and alveolar fluid accumulation usually develops rapidly after acute injury to the central nervous system (1), and any preventive treatment should be given early after BD (3). This lung injury mimics a form of acute respiratory distress syndrome (ARDS), the most devastating form of acute lung injury (ALI), with excessive intra-alveolar flooding. Although NPE and ARDS use different mechanisms in the development of ALI, they share a common downstream phenotype that targets the disruption of vascular endothelial cell barrier integrity. Well-defined pulmonary lesions are described in cases of BD during elevated intracranial pressure (ICP), and represent an important cause of transplantation failure (5).

Sphingosine 1-phosphate (S1P) is a sphingolipid angiogenic factor and the metabolic product of sphingomyelinase activity, and represents a vitally important signaling mediator with potent vascular barrier-regulating properties (6–8). Our *in vitro* and *in vivo* studies indicate that the intravenous administration of S1P attenuates inflammatory lung injury (9–11) and vascular permeability, via the ligation of the G-protein-coupled S1P receptor S1PR1 (8, 12, 13). We also demonstrated that S1P attenuates ischemic/reperfusion-induced ALI in rodents (14) and, via S1PR1 receptor ligation, is essential for platelet-mediated barrier enhancement (13). Because primary lung graft dysfunction is characterized by a loss of vascular endothelial cell barrier function, we hypothesized that the use of barrier-enhancing agents such as the S1PR1 agonist SEW-2871 (15) could attenuate NPE when administered to a lung donor shortly after BD. In the present study, we explored the effects of SEW-2871 on neurogenic pulmonary injury after BD in a rat model.

MATERIALS AND METHODS

Animal Care and Reagents

These experiments were approved by the Animal Care and Use Committees of the University of Chicago and the University of Illinois at Chicago. Male Sprague-Dawley (SD) rats (weighing 250–350 g) were purchased from Charles River (Wilmington, MA) and housed with free access to food and water in a temperature-controlled room. We used 4–6 rats per group. SEW-2871 was purchased from Cayman (Ann Arbor, MI).

Experimental Model

A midline incision was made through the scalp. A burr hole was drilled to expose the dura mater on intubated and ventilated rats after anesthesia for 4 hours, using an Inspira ventilator (Harvard Apparatus, Boston,

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MA) at a 12 ml/kg tidal volume. The common carotid artery was cannulated with an intravenous catheter and connected to Powerlab (AD Instruments, Colorado Springs, CO) to obtain the mean arterial main blood pressure (MABP). A Fogarty catheter (4 French) was inserted and secured in the extradural space, and inflated to induce BD by increasing ICP and brainstem herniation in the BD group. SEW-2871 (0.1 mg/kg) was given intravenously to the treated group 15 minutes or 2 hours after brain death, and to sham-operated non-BD rats. The non-BD group ($n = 6$) received saline 15 minutes after the beginning of ventilation. Temperature was maintained using a warming pad connected to a temperature-controlled system. Bronchoalveolar lavage (BAL) fluid was collected from the left lung, after ligating the right bronchus for assessments. Lungs were used for the determinations of wet/dry weight (W/D) ratios or histology.

Accumulation of BAL Protein and Leukocyte Quantification

BAL was performed by flushing the lungs with 5 ml of cold Hanks' balanced salt solution (Invitrogen, Grand Island, NY) via the tracheal cannula, as previously described (16). Total cells were counted with a hemacytometer. Differential counts of BAL cells were determined via cytocentrifugation (Cytospin 3; Shandon Instruments, Pittsburgh, PA), and BAL cells were stained with Diff-Quik (Dade Behring, Düringen, Switzerland).

Measurements of BAL Total Protein, Albumin, and Concentrations of IL-6

Protein concentrations in BAL fluid were measured using an RC DC Protein Assay (Bio-Rad, Hercules, CA), as previously described (16, 17). Sample readings (optical density) were converted to mg/ml, using albumin as standard (0.1–1.5 mg/ml). Concentrations of albumin in BAL were quantified, using a sandwich ELISA (Bethyl Laboratories, Montgomery, TX). A Bioplex kit (Bio-Rad, Hercules, CA) was used to measure concentrations of IL-6 in BAL.

Lung Histopathology

Portions of the left and right middle lobes were fixed in 100% formalin and embedded in glycol methacrylate (10). Sections (5–6 μ m thick) were stained with hematoxylin-and-eosin for evidence of inflammation and injury.

RNA Isolation and Microarray

Total lung RNA was extracted with a TRIzol/RNeasy kit (Invitrogen, Carlsbad, CA), as previously reported (18). Gene expression analysis was performed with Affymetrix Rat expression set 230_2. Genes differentially expressed across groups were determined using the Significance Analysis of Microarrays (SAM) (19). The Self-Organizing Map was performed by the GeneCluster2 software (Broad Institute, Cambridge, MA) (20).

Statistical Analysis

Except where noted, results were analyzed using standard one-way ANOVA. Group differences were compared by the Newman-Keuls test. $P < 0.05$ was considered significant. Details are described in the online supplement.

RESULTS

Effect of BD on MABP

The autonomic storm accompanying BD in a group of male SD rats led to a dramatic and rapid increase in MABP from approximately 80 mm Hg to approximately 140 mm Hg within 1 minute after the induction of BD by intracranial balloon inflation. MABP values subsequently declined rapidly, to levels below the primary recorded MABP values (~ 60 mm Hg) before stabilization. Sham-operated, non-BD control animals maintained

a relatively stable MABP of approximately 80 mm Hg during the entire procedure (Figure 1). This dramatic increase in blood pressure was induced by the acute increase in ICP after the balloon inflation and induction of brainstem herniation leading to BD, and was accompanied by a total loss of cranial-nerve reflexes, absolute apnea, and maximal pupillary dilation.

BD-Induced ALI

To assess the role of BD in lung barrier function, a group of male SD rats ($n = 6$) was placed on mechanical ventilation (Harvard Apparatus) before inducing BD, and was subsequently monitored for 4 hours (BD group) and compared with ventilated sham rats without BD challenge ($n = 6$). Both groups were injected intravenously with 500 μ l of saline. The BD group exhibited evidence of ALI, with significant increases in BAL cellularity ($*P = 0.0015$) (Figure 2) and enhanced microvascular permeability, as reflected by significant increases in BAL total protein (Figure E1 in the online supplement; $*P = 0.001$) and BAL albumin (Figure 3, $*P = 0.007$), compared with the sham non-BD group. Moreover, BAL concentrations of proinflammatory cytokine (IL-6) were also significantly increased in BD rats compared with sham-treated rats (Figure 4, $*P = 0.0009$). The disruptive effects of BD on barrier function as induced via lung edema were reflected by a significant increase in the lung tissue W/D weight ratio after BD, compared with the sham group (Figure 5, $*P = 0.012$).

Effect of SEW-2871 on BD-Induced Lung Injury

To evaluate the potential attenuation of BD-induced lung injury and barrier dysfunction by SEW-2871, male SD rats were treated with 0.1 mg/kg SEW-2871 (intravenous) delivered 15 minutes after the induction of BD, and lungs were harvested at the end of 4 hours. BAL cellularity was significantly reduced in SEW-2871-treated BD rats compared with vehicle-treated BD rats ($\sim 50\%$) (Figure 2, $**P = 0.02$). Similarly, BAL total protein (Figure E1, $**P = 0.0001$) and BAL albumin concentrations (Figure 3, $**P = 0.01$) were significantly decreased by treatment with SEW-2871. SEW-2871 (intravenous; 0.1 mg/kg) was also

Figure 1. Effect of brain death (BD) on mean arterial blood pressure (MABP). The carotid artery was cannulated in rats of all study groups, and MABP was monitored for the duration of the procedure, using an intravenous catheter connected it to an AD instrument acquisition system (AD Instruments, Colorado Springs, CO). After total inflation of the balloon inserted in the intracranial space, MABP instantly began to rise dramatically, reaching approximately 140 mm Hg ($*P = 0.001$) and then declining rapidly to values of approximately 65 mm Hg before stabilization. The MABP spike was not observed in sham-operated, non-BD rats, in whom stable MABP was observed during the 4-hour period.

Figure 2. Treatment with SEW-2871 reduces the BD-induced accumulation of leukocytes (WBCs) in bronchoalveolar lavage (BAL) fluid. Fluid was collected 4 hours after BD, and total numbers of WBCs were counted using a hemacytometer. A significant increase in the BAL WBC count was evident in the BD group compared with the control group ($*P = 0.0015$). BD-mediated cell infiltration into the BAL was reduced by an intravenous administration of SEW-2871 ($**P = 0.02$). Values in the control group with SEW-2871 alone were not significantly different from those in the sham-operated non-BD rats.

tested in mechanically ventilated rats without BD (4 hours), and resulted in minimal alterations in BAL total inflammatory cell count and BAL total protein concentrations, compared with concentrations in the sham group. Concentrations of the proinflammatory cytokine, IL-6, were also significantly reduced in BD rats treated with SEW-2871 compared with sham-treated rats (Figure 4, $**P = 0.001$). The attenuation by SEW-2871 of BD-increased vascular leakage and neurogenic lung edema was reflected by the significant decrease in the lung tissue W/D weight ratio in the SEW-2871-treated BD rats compared with the untreated rats with BD (Figure 5, $**P = 0.0002$).

Histological Evaluation of Effects of SEW-2871 on BD-Induced Rodent Lung Injury

The histological assessment of lung tissue after BD revealed an infiltration of interstitial inflammatory cells (neutrophils, i.e., polymorphonuclear leukocytes), with evidence of edema without

Figure 3. SEW-2871 ameliorates BD-induced lung vascular permeability in Sprague Dawley (SD) rats. In BD-challenged SD rats, BAL albumin content (an indicator of lung vascular permeability) was elevated compared with that in sham-operated non-BD rats ($*P = 0.007$). The intravenous administration of SEW-2871 (0.1 mg/kg) shortly after BD attenuated BD-induced lung barrier disruption, and dramatically reduced BAL albumin concentrations, compared with the saline-injected BD rats ($**P = 0.01$). Rats receiving SEW-2871 alone did not exhibit evidence of altered BAL albumin concentrations.

Figure 4. Barrier-enhancing properties of SEW-2871 on BD-induced lung barrier function in BD rats were reflected by concentrations of IL-6 in BAL fluid. BD was associated with an approximately 50% increase in concentrations of BAL cytokine (IL-6) ($*P = 0.0009$), compared with sham-operated non-BD rats. BD-induced increases in IL-6 concentrations were reduced by an intravenous administration of SEW-2871 (0.1 mg/kg) by approximately 60% ($**P = 0.001$). In sham-operated non-BD rats, SEW-2871 alone exerted minimal effects on IL-6 concentrations in BAL.

the formation of hyaline membranes and greater alveolar wall thickening compared with the normal histological lung structure observed in sham rats or SEW-2871-treated, non-BD rats (Figures 6A and 7B). Lung tissue from SEW-2871-treated rats with BD demonstrated a remarkable attenuation of the inflammatory process, with a reduced accumulation of polymorphonuclear leukocytes, less formation of edema, and thinner alveolar walls, compared with untreated BD rats (Figure 6C and 6D). The average infiltrative quantification demonstrated the relative number of infiltrative inflammatory cells in lung tissue, and reflected the remarkable barrier-enhancing properties of SEW-2871 on BD-induced lung injury (Figure 6E, $**P = 0.02$).

Figure 5. BD-induced lung edema is attenuated by intravenous administration of SEW-2871. At the end of the experiment, lungs were harvested, and the weight of the accessory lobe was recorded as a wet weight. This lobe was then directly placed in an oven at 70°C and kept for 3 days before ascertaining the dry weight, to determine the wet/dry (W/D) weight ratio as an index of BD-induced lung edema. A significant increase in the W/D ratio was evident in the BD group compared with the sham-treated non-BD group. The administration of SEW-2871 (0.1 mg/kg) 15 minutes after BD significantly decreased this ratio ($*P = 0.012$). BD-induced lung edema, reflected as W/D weight ratio, was reduced by an administration of SEW-2871 (0.1 mg/kg) 4 hours after injury ($**P = 0.0002$). Overall, SEW-2871 alone exerted minimal effects on lung W/D weight ratio in sham-operated non-BD rats.

Figure 6. Histological evaluation of the barrier-enhancing properties of SEW-2871 on BD-induced lung injury in SD rats. Tissue samples were obtained from both right and left lungs at the end of the procedure. Sections were prepared after fixation with 10% formalin and stained with hematoxylin-and-eosin. (A and B) Normal lung tissue structure in samples from the sham-operated non-BD group and the SEW-2871–tested non-BD group, respectively. (C) Diffuse neutrophilic (i.e., polymorphonuclear leukocytes) infiltration into the lung tissue of BD-challenged SD rats, with the formation of lung edema and an increase in alveolar wall thickness. (D) The barrier-enhancing properties of SEW-2871 in the lungs of a BD rat, with a remarkable decline in the accumulation of neutrophils, milder lung edema, and a thinner alveolar wall, compared with the BD group (E),

hence demonstrating a relative average quantification of infiltrated inflammatory cells in the lung tissue in all study groups, and reflecting the remarkable barrier-enhancing properties of SEW-2871 on BD-induced lung injury (* $P = 0.003$, compared with the sham-operated group; ** $P = 0.02$, compared with BD-injured rats).

Microarray Analysis of BD-Induced Gene Dysregulation: Attenuating Effects of SEW

Microarray data were submitted to the Gene Expression Omnibus repository of the National Center for Biotechnology Information (accession number GSE22531). Whole-lung, genome-wide levels of gene expression were analyzed, and differentially expressed genes across four groups were identified using SAM software, with false discovery rate of less than 0.05. The list of 3,670 genes is accessible at www.phenogo.org/publication/BrainDeath-SEW. This gene list was then submitted to GeneCluster2 software, to construct diverse expression clusters based on the mean of gene ranks across samples. In the majority of gene clusters (7/12 clusters, or 2,434/3,670 genes), SEW displayed an attenuation of BD-induced gene dysregulation (Figure 7A). The 201 genes in Cluster 5 (C5) were used to construct heat maps, facilitating the visualization of expression levels across experimental groups. All C5 genes were up-regulated by BD but down-regulated by SEW, with the majority of these genes in the BD–SEW group demonstrating near-normal expression levels, indicating the attenuation by SEW of BD-induced gene dysregulation (Figure 7B). The functional enrichment of C5 genes with Ingenuity software (Ingenuity Systems, Inc., Redwood City, CA) identified several significant canonical signaling pathways involved in immune processes and inflammation (Figure 7C), as well as gene networks regulated by TNF and NF- κ B (Figures E2A and E2B, respectively). The expression of genes up-regulated by BD but down-regulated by SEW, that is, TNF and CCRL2, was further validated by quantitative PCR (Figure 7D, with genes listed in Table E1).

DISCUSSION

Lung transplantation is an accepted form of therapy for selected patients with endstage lung disease. However, despite stringent criteria for the selection of potential recipients, a far greater number of patients await transplantation compared with the availability of donor lungs. This imbalance results in significant mortality for patients on the lung transplant waiting list, and is a major limiting factor of lung transplantation as an effective form of therapy in endstage lung disease. Only 20% of all multi-organ cadaveric donors are used as lung donors worldwide (21), and the vast majority of donor lungs for transplantation are obtained from heart-beating BD donors (21, 22). BD is associated with significant hemodynamic instability because of the

well-studied autonomic storm associated with BD (23, 24), which results in increased alveolar capillary leakage and NPE, triggering a systemic and pulmonary inflammatory response in the donor (1–3). The neurogenically induced hypertension with subsequent hypotension associated with the autonomic storm further amplifies the inflammatory responses that contribute to lung injury (1, 5, 25).

The mechanism of lung injury in BD donors is poorly understood, and the inability to predict lung graft injury during donor selection is a root cause of pulmonary graft dysfunction (PGD) in lung transplant recipients. Furthermore, the current criteria of clinical history, physical examination, radiological findings, blood gas analysis, and bronchoscopic evaluation are inadequate in predicting PGD, whose incidence ranges from 15–75%, sometimes resulting in graft failure and mortality. Early lung injury appears to serve as a precursor for late graft failure. The attenuation of lung injury related to donor BD may therefore constitute a reasonable contributor in preventing subsequent injury to the graft.

S1P, a byproduct of sphingomyelin metabolism, is a potent vascular barrier–protective molecule (8–10, 14), and is produced by nearly all cell types but is particularly in abundance in platelets because of their lack of sphingosine lyases. S1P binds to the S1P-specific G-protein–coupled S1PR1 receptor on the endothelial cell surface, resulting in cytoskeletal rearrangement and reduced agonist-induced permeability (6–8, 26, 27). S1PR1 receptors signal through Gi protein to promote recruitment into membrane lipid rafts, and Gi-coupled signaling to cytoskeletal elements via the small GTPase Ras-related protein family results in vascular maturation and decreased permeability (8, 11). We previously showed that S1P protects lungs from injury during ischemia reperfusion, ventilator-induced lung injury, and lung injury caused by endotoxin (6, 14, 15). We also used S1P analogues such as 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (or FTY720) (28) and SEW-2871 (29) as S1PR1 agonists to evaluate the protective effects of S1P in lung injury. Because endothelial cell barrier disruption is a common downstream phenotype of both ARDS and NPE, S1P may represent an excellent candidate to attenuate the lung injuries observed in these models.

In the present study, SEW-2871 produced substantial protective effects in BD-induced NPE, with reductions in BAL total protein content and BAL leukocytes and W/D weight lung

Figure 7. Identification of gene sets reflecting BD-induced gene dysregulation. Genes differentially expressed across the four experimental groups were identified by one-way ANOVA, with false discovery rate less than 0.05. (A) The genes were classified by the Self-Organizing Map to retrieve those with opposite patterns of expression between BD and SEW. The order of experimental groups is Control, BD, SEW, and BD-SEW in each cluster. Cluster C5 (A, yellow highlight) revealed the counter-effect of SEW in BD up-regulated genes. (B) Levels of gene expression in the BD-SEW group are close to those in the normal control group (CTR), as shown in the heat map constructed from these genes. (C) Functional enrichment of C5 genes was analyzed using Ingenuity Pathway Analysis software. (D) Quantitative PCR (qPCR) validation of the differential expression of both chemokine CC motif receptor-like 2 and TNF. THOP1 = thimet oligopeptidase 1; RIG-1 = RLR(RIG-1 like Receptors); Wnt/ β -catenin = wingless type/beta-catenin; Oct4 = octamer binding protein; PI3/AKT = phosphoinositide-3-kinase/V-AKT murine thymoma viral oncogene homolog.

ratios. Furthermore, SEW attenuated the BD-induced increases in BAL concentrations of the proinflammatory IL-6 and histological lung abnormalities. The effects of SEW-2871 on non-lung organ systems were not evaluated after BD, but are currently under study. SEW and S1P agonists improve endothelial capillary dysfunction in multiple vascular beds, and we speculate that SEW will improve vascular function in these organs as well. Due to early, profoundly injurious hemodynamic changes, we reasoned that injecting SEW-2871 15 minutes after the induction of BD would be beneficial, whereas its injection at later time points would have a very limited effect on lung injury.

SEW-2871 was tested in a group of SD rats ($n = 5$) 2 hours after BD, but no physiological improvement was detected compared with the sham-treated group (Figure E3).

In this study, we also used genomic approaches to decipher the effects of BD on lung gene expression, as well as the effects of SEW on BD-mediated gene dysregulation. We noted that BD and SEW exert diverse impacts on gene expression. Among the 3,670 genes differentially expressed across the four experimental groups, the majority of genes (66%) displayed a reversal of BD-driven expression by SEW. The expression levels in a small set of 201 genes up-regulated by BD returned

in the BD–SEW group to near-normal control levels, thus revealing the potential molecular mechanism underlying the therapeutic effects of SEW, along with the main biological function of these genes involved in immune and inflammatory responses.

In conclusion, we used a rat model of BD intimately related to lung transplantation, and investigated the potential therapeutic effects of SEW-2871 in the donor lung after BD, to imitate the clinical scenario. Our results indicate that the acute increases in ICP associated with BD produce significant inflammatory lung injury and increases in vascular permeability, which were attenuated by SEW-2871, an S1PR1 agonist. These findings are significant for the preservation of organs after BD in donor cadavers, to combat the universal shortage of suitable lungs for transplantation.

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