Specialized pro-resolving lipid meditators agonistic to formyl peptide receptor type 2 attenuate ischemia-reperfusion injury in rat lung

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Authorship statement

H.O. conceived the primary hypothesis, designed the research, collected and analyzed the data, performed the experiments and histopathological evaluations, and wrote the manuscript; S.T. conceived the primary hypothesis, designed the research, analyzed the data, and wrote the manuscript; M.S. collected and analyzed the data and wrote the manuscript; Y.M Y.Yokoyama performed histopathological evaluations; H.K., Y.Yamada, Y.Yutaka, A.O., D.N., M.H., and T.M. contributed to the methodology and edited the manuscript; H.D. supervised the research and edited the manuscript.

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Abbreviations

15-HETE, 15-hydroxy eicosatetraenoic acid

17-HDHA, 17-hydroxy docosahexaenoic acid

5-HETE, 5S-hydoxy-eicosatetraenoic acid

AA, arachidonic acid

ALX/FPR2, formyl peptide receptor type 2

AT, aspirin-triggered

AT-LXA$_4$, asprin-triggered lipoxin A$_4$

AT-RvD1, asprin-triggered resolvin D1

ChemR23, chemerin receptor 23

d, deuterium

DHA, docosahexaenoic acid

ELISA, enzyme-linked immunosorbent assay

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GPR18, G protein-coupled receptor 18

HPFs, high-power fields

IL, interleukin

IRI, ischemia-reperfusion injury

LC–MS/MS, liquid chromatography–tandem mass spectrometry

LM, lipid mediator

LXA$_4$, lipoxin A$_4$

LXs, lipoxins

MPO, myeloperoxidase

MRM, multiple-reaction monitoring

pO$_2$, partial pressure of oxygen

PGD, primary graft dysfunction

qRT-PCR, quantitative reverse transcription polymerase chain reaction

R, reperfusion

RvD1, resolvin D1

RvDs, D-series resolvins

RvEs, E-series resolvins

SDC, supplemental digital content

SPMs, specialized pro-resolving lipid mediators

TNF-α, tumor necrosis factor - α

W/D, wet to dry weight

WI, warm ischemia

Abstract

Background: Lung ischemia-reperfusion injury (IRI) is a form of acute lung injury characterized by non-specific alveolar damage and lung edema due to robust inflammation. Little is known about the roles of specialized pro-resolving lipid mediators (SPMs) in lung IRI. Therefore, we aimed to evaluate the dynamic changes in endogenous SPMs during the initiation and resolution of lung IRI and to determine the effects of SPM supplementation on lung IRI.

Methods: We used a rat left hilar clamp model with 90 min of ischemia, followed by reperfusion. Dynamic changes in endogenous SPMs were evaluated using liquid chromatography–tandem mass spectrometry.

Results: Endogenous SPMs in the left lung showed a decreasing trend after 1 h of reperfusion. Oxygenation improved between 3 and 7 days following reperfusion; however, the level of endogenous SPMs remained low compared with that in the naïve lung. Among SPM receptors, only formyl peptide receptor type 2 (ALX/FPR2) gene expression in the left lung was increased 3 h after reperfusion, and the inflammatory cells were immunohistochemically positive for ALX/FPR2. Administration of aspirin-triggered (AT) resolvin D1 (AT-RvD1) and AT-lipoxin A_4 (AT-LXA$_4$), which are agonistic to ALX/FPR2, immediately after reperfusion improved lung function, reduced inflammatory cytokine levels, attenuated lung edema, and decreased neutrophil infiltration 3 h after reperfusion. The effects of AT-RvD1 and AT-LXA$_4$ were not observed after pretreatment with the ALX/FPR2 antagonist.

Conclusions: The level of intrapulmonary endogenous SPMs decreased during lung IRI process and the administration of AT-RvD1 and AT-LXA$_4$ prevented the exacerbation of lung injury via ALX/FPR2.

Introduction

Lung transplantation is a life-saving surgery performed in patients with end-stage lung disease. Despite advances in perioperative management and organ preservation, primary graft dysfunction (PGD) in the early stages of lung transplantation is observed in approximately 30% of patients.[1] It has been reported that PGD is the major cause of perioperative death and a risk factor for chronic rejection.[2,3] Multiple factors have been reported to be involved in PGD,[1,4] including ischemia-reperfusion injury (IRI). There are several studies on preventing lung IRI;[5,6] however, there is no effective therapeutic strategy. Lung IRI is a form of acute lung injury characterized by non-specific alveolar damage and pulmonary edema due to epithelial and endothelial dysfunction[7,8] with sterile acute inflammation.[9] Lung IRI also occurs in clinical conditions, such as trauma,[10] resuscitation for circulatory arrest,[10] and pulmonary embolism.[11]

It has been believed that the inflammatory response induced after ischemia or infection is an active mechanism and that the resolution of inflammation is a passive response. However, recent studies have shown that the resolution of inflammation is an active process caused by the biosynthesis of active mediators that promote the return to homeostasis.[12,13] Lipid mediators, which are involved in this process to actively resolve inflammation, are specialized pro-resolving lipid mediators (SPMs).[13] D-series resolvins (RvDs), protectin D1, and maresin 1 and 2 derived from docosahexaenoic acid (DHA), E-series resolvins (RvEs) derived from eicosapentaenoic acid, and lipoxins (LXs) derived from arachidonic acid (AA) have been identified as SPMs.[14] SPMs have been reported to play pivotal roles in inflammatory response, from initiation to resolution.[15] SPMs suppress the infiltration of polymorphonuclear cells at the initiation phase of

inflammation,[16] and stimulate the recruitment of non-phlogistic monocytes[17] and resolving macrophages,[18] leading to the resolution of inflammation. Moreover, SPMs exert their biological actions via the activation of cognate G protein-coupled receptors and signaling pathways.[19–22]

It is assumed that SPMs play an important role in the initiation and resolution of lung IRI; however, to date no reports have shown the dynamic changes in endogenous SPMs throughout the process of lung IRI. In addition, dynamic changes in SPMs and their receptors in the process of lung IRI have not yet been evaluated.

In this study, we aimed to evaluate the dynamic changes in endogenous SPMs and the expression of SPM receptors in the process of lung IRI. Further, we examined the hypothesis that SPMs have protective effects via their cognate receptor against lung IRI.

Materials and Methods

Animals

Male Lewis rats, aged 9–11 weeks and weighing 270–300 g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All the rats were inbred and housed in a specific pathogen-free barrier facility. All animal protocols were approved by the Institutional Animal Care and Use Committee of Kyoto University (Medkyo 20292).

Rat left hilar clamp model

We used the previously reported rat lung IRI model[23] with modifications as described in the supplemental digital content (SDC). Briefly, each rat was anesthetized and heparinized. After thoracotomy, the left pulmonary hilum was clamped. Following 90 min of warm ischemia (WI), the clamp was removed and reperfusion was allowed to take place. The rats were sacrificed for evaluation at the indicated time points. We evaluated the dynamics of endogenous SPMs, SPM receptors, effect of SPM administration on lung IRI, and effects of combined administration of SPMs and SPM receptor antagonist on lung IRI.

Assessment of oxygenation and lung function

During the assessment of oxygenation and lung function, the right pulmonary hilum, including the accessory lobe, was occluded with a vascular clamp under median sternotomy, and the left lung was ventilated with a tidal volume of 5 mL/kg and

respiratory rate of 70 breaths/min. The fraction of inspired oxygen was maintained at 1.0. Five min after occlusion of the right hilum, blood sample was collected through the ascending aorta for arterial blood gas analysis.

The rats were moved from a normal ventilator to a rodent ventilator (flexi Vent, SCIREQ, Montreal, Quebec, Canada) to measure left pulmonary function. The dynamic compliance, mean airway pressure, and peak airway pressure were measured as previously described.[23,24]

Lung wet to dry weight ratio

After the measurement of pulmonary function, the left lung was harvested to obtain tissue samples and was divided into three parts. The lower part of the left lung was used to calculate the lung wet to dry weight (W/D) ratio. The wet weight was measured immediately after harvesting, whereas the dry weight was measured after 24 h of desiccation at 100 °C. The ratio was calculated as the wet weight/dry weight.

Lipid mediator (LM) lipidomics

The left lung was frozen, and LM lipidomics was performed as described previously.[25] Deuterium (d)-labeled internal standards, d_8-5S-hydoxy-eicosatetraenoic acid (5-HETE), d_4-leukotriene B_4, d_5-LXA$_4$, d_4-prostaglandin E_2, and d_5-RvD2 in ice-cold methanol were added to facilitate the quantification of sample recovery. All samples for LM lipidomics were extracted using C18 solid-phase extraction columns and were subject to liquid

chromatography–tandem mass spectrometry (LC–MS/MS). The LC–MS/MS system, QTrap 6500 (AB Sciex, Framingham, MA, USA), was equipped with a Nexera X2 HPLC system (Shimadzu, Kyoto, Japan). An Agilent Eclipse Plus C18 column (100 mm × 4.6 mm × 1.8 µm) was used with a gradient of methanol:water:acetic acid of 55:45:0.01 (v:v:v) to 98:2:0.01 at a flow rate of 0.4 mL/min. To monitor and quantify the levels of the targeted LM, the multiple-reaction monitoring (MRM) method was devised with signature ion fragments for each molecule. Identification was performed using published criteria,[25] LC retention times, specific fragmentation patterns, and at least six diagnostic fragmentation ions. Quantification was performed based on the peak area of the MRM transition, and linear calibration curves were obtained using the authentic standard for each compound. The standard for protectin D1 was kindly provided by Professor Charles N. Serhan (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA). The standard for RvE3 was kindly provided by Professor Makoto Arita (Keio University, Tokyo, Japan). The other standards were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The upper part of the left lung was used to perform qRT-PCR to evaluate the gene expression of SPM receptors such as formyl peptide receptor type 2 (ALX/FPR2), G protein-coupled receptor 18 (GPR18), and chemerin receptor 23 (ChemR23), as described in the SDC.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in the upper part of the left lung tissue were evaluated using by performing ELISA, as described in the SDC.

Histological analysis

The middle part of the left lung was fixed in 10% formalin and embedded in paraffin. The tissue sections were de-paraffinized and stained with hematoxylin and eosin.

The index of perivascular cuff area to the vessel area was calculated as reported previously.[26] The perivascular cuff area was measured for five vessels per rat using ImageJ version 1.53a Java 1.8.0 (National Institutes of Health, Bethesda, MD, USA). The index was calculated to eliminate variations related to vessel size.

The number of red blood cells in the alveolar space was expressed as the average number of five randomly chosen high-power fields (HPFs) per section at a magnification of 400×, as determined by automatic cell counting using the ImageJ software.

For ALX/FPR2 (1/1500 dilution, Novus Biologicals, Centennial, CO, USA) and myeloperoxidase (MPO) (1/150 dilution, Abcam, Cambridge, UK) immunohistochemical staining, the tissue sections were de-paraffinized and incubated overnight at 4 °C. MPO immunohistochemical staining is used for detecting neutrophils;[27] in this study, it was used to count the neutrophils infiltrating the lung. The number of neutrophils was expressed as the average number of extravascular neutrophils in five randomly chosen

HPFs per section at a magnification of 400×. Three separate investigators (H.O., Y.M., and Y.Yokoyama) performed the evaluation in a blinded manner.

Statistical analyses

Data are presented as medians with interquartile ranges, unless otherwise indicated. Histological results are presented as box and whisker plots. Kruskal-Wallis nonparametric analysis followed by Dunn's multiple comparison tests were used to evaluate the differences among the experimental groups. Statistical significance was set at P < 0.05. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

Results

Dynamics of endogenous SPMs during the onset and resolution process of lung IRI

First, we evaluated the time course of the initiation and resolution process of lung IRI using the left hilar clamp model (n = 4/group) (Figure 1A). Peak and mean airway pressure, compliance, and the partial pressure of oxygen (pO_2) were the worst at 12 h after reperfusion (Figure 1B–1E), and W/D ratio was the highest at 3 h after reperfusion (Figure 1F); however, most of the parameters improved thereafter. LM lipidomics revealed the concentrations of endogenous SPMs in the damaged left lung at different time points following reperfusion (n = 4/group) (Figure 2A–2I). SPMs including RvD1, protectin D1, and maresin 1 and 2 derived from DHA are shown in Figure 2A–2D, while

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RvE3 derived from EPA is shown in Figure 2E. LXA$_4$ and LXB$_4$ derived from AA are shown in Figure 2F and 2G. RvD2, RvD3, RvD5, LXA$_5$, RvE1, and RvE2 concentrations were less than the detection limit at all the time points. MS/MS spectrum of RvD1 and LXA$_4$ are shown in Figure 2J and 2K. Others are presented in the SDC (Figure S1A–S1G).

Each reperfusion group was statistically compared with naïve group at each time point. The concentration of each endogenous SPMs decreased after 1 h of reperfusion. Surprisingly, they continued to be low, even after oxygenation, whereas other parameters improved. In particular, the endogenous RvD1 and LXA$_4$ levels were substantially lower at 3 h, 12 h, and 7 days after reperfusion compared to those in naïve lung, although the levels of resolvin precursor 17-hydroxy docosahexaenoic acid (17-HDHA) and lipoxin precursor 15-hydroxy eicosatetraenoic acid (15-HETE) recovered 7 days after reperfusion (Figure 2H, 2I).

In addition, we also evaluated neutrophil infiltration in the lungs at each time point when the endogenous SPMs were evaluated (Figure S2). The neutrophil infiltration level was high up to 3 h after reperfusion, but decreased thereafter. Based on these results, we estimated that the appropriate time for the evaluation of initiation phase of lung IRI was after 3 h of reperfusion.

Identification of upregulated SPM receptor in lung IRI

Next, we evaluated the expression and localization of SPM receptors in the initiation phase of lung IRI (Figure 3A). Gene expression of ALX/FPR2, GPR18, and ChemR23 in

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the left lung tissue were compared with the naïve lung, lung with WI only, and lung with WI followed by 3 h of reperfusion, respectively (n = 8/group) (Figure 3B–3D). The mRNA expression of ALX/FPR2 did not increase in the lung with WI only, compared with the naïve lung, which significantly increased after 3 h of reperfusion (Figure 3B; $P > 0.99$ and $P < 0.01$, respectively). Furthermore, compared with the naïve lung, GPR18 mRNA and ChemR23 mRNA did not show increased expression following WI or WI and reperfusion (Figure 3C, 3D). Immunohistochemistry analysis revealed that ALX/FPR2 was positive in the airway epithelium and inflammatory cells, such as granulocytes, monocytes, and macrophages (Figure 3E).

The effects of SPMs agonistic to ALX/FPR2 on lung function in lung IRI

Based on the aforementioned findings from the dynamic changes of endogenous SPMs and their receptors in the initiation phase of lung IRI, we hypothesized that the supplementation of SPMs agonistic to ALX/FPR2 after reperfusion would attenuate lung IRI. Among SPMs, RvD1 and LXA_4 are agonists of ALX/FPR2. Aspirin-triggered (AT) epimers such as AT-RvD1 and $AT-LXA_4$ were used for this experiment as they have higher stability, which is transformed in the presence of aspirin.[28–30] They also exert biological effects via ALX/FPR2.[19,31–33] AT-RvD1, $AT-LXA_4$, or its vehicle was administered immediately after reperfusion in the rat hilar clamp model (n = 8/group). We evaluated lung function after 3 h of reperfusion (Figure 4A). The vehicle group was statistically compared to each administration group. Peak and mean airway pressures were lower and dynamic compliance and pO_2 were higher in the AT-RvD1 and $AT-LXA_4$ groups than that in the vehicle group (Figure 4B–4E; $P < 0.01$). The results of the groups

that underwent reperfusion for more than 3 h are presented in the SDC (Figure S3A, S3B). The effects of AT-RvD1 and AT-LXA$_4$ diminished as reperfusion time increased.

The effects of AT-RVD1 and AT-LXA$_4$ on cytokine level, lung edema, alveolar hemorrhage, and neutrophil infiltration in lung IRI

Administration of AT-RvD1 and AT-LXA$_4$ immediately after reperfusion decreased inflammatory cytokine levels in the lung tissue (Figure 5A–5C). ELISA of lung tissue lysates revealed that the levels of IL-1β and IL-6 were significantly decreased in the AT-RvD1 and AT-LXA$_4$ groups than those in the vehicle group (n = 8/group) (Figure 5A; P = 0.038 and P < 0.001, respectively and Figure 5B; P = 0.021 and P < 0.001, respectively). The level of TNF-α was significantly decreased in the AT-LXA$_4$ group (Figure 5C, P = 0.046), while the level of TNF-α tended to be lower in the AT-RvD1 group than that in the vehicle group (Figure 5C, P = 0.179). The findings of hematoxylin and eosin staining and immunohistochemical staining of MPO of each group are shown in Figure 5D and 5E, respectively. Lung edema was attenuated in the AT-RvD1 and AT-LXA$_4$ groups, as demonstrated by the lower W/D ratio (Figure 4F) and the decreased perivascular cuff area (Figure 5F). The number of red blood cells (Figure 5G; P < 0.001) and neutrophil infiltrations (Figure 5H; P < 0.001) in the alveolar area was significantly lower in the AT-RvD1 and AT-LXA$_4$ groups.

The effects of AT-RvD1 and AT-LXA$_4$ on lung IRI after the pretreatment with ALX/FPR2 antagonist

Next, we confirmed the protective effects of AT-RvD1 and AT-LXA$_4$ via ALX/FPR2. In this experiment, the ALX/FPR2 antagonist WRW4 was administered prior to AT-RvD1 or AT-LXA$_4$ supplementation. Lung function and histological findings were evaluated 3 h after reperfusion (n = 8/group) (Figure 6A). The WRW4+vehicle group and each of the WRW4-pretreated AT-RvD1 and AT-LXA$_4$ supplementation groups were statistically compared. After pretreatment with WRW4, neither AT-RvD1 nor AT-LXA$_4$ groups showed significant differences in peak or mean airway pressures, dynamic compliance, pO$_2$, or W/D ratio (Figure 6B–6F; $P > 0.05$). Histological findings were similar among the vehicle, AT-RvD1, and AT-LXA$_4$ groups after pretreatment with WRW4 (Figure 6G).

Discussion

In this study, we demonstrated the following findings in a rat model of lung IRI. First, endogenous SPMs, including RvD1 and LXA$_4$, tended to decrease immediately after reperfusion and remained low even after the recovery of lung function. Second, ALX/FPR2 expression was upregulated in the initiation phase of IRI lung. Third, AT-RvD1 and AT-LXA$_4$ prevented exacerbation of lung IRI via ALX/FPR2.

SPMs are endogenously produced in vivo and are involved in the initiation and resolution of inflammation.[13,15–18] Studies have reported their effects on various lung injury models.

RvD1 suppressed inflammatory cytokines and cell infiltration, and alleviated smoking-induced emphysema in mice.[34,35] Further, AT-RvD1 reduced neutrophil elastase activity, restored total antimicrobial activity, and reduced the number of infiltrating lung neutrophils and monocytes/macrophages in a mouse model of

pneumonia.[36] AT-RvD1 inhibits neutrophil-platelet heterotypic interactions in murine acid-induced acute lung injury.[37] LXA4 promotes epithelial cell proliferation, decreases epithelial cell apoptosis, and inhibits epithelial–mesenchymal transition,[38] while AT-LXA4 induces NF-κB regulation,[39] which has been reported to alleviate acid or lipopolysaccharide (LPS)-induced acute lung injury in mice.

Lung IRI is underlined with sterile inflammation caused by monocytes and neutrophils infiltrating the lung.[9,40] Based on these findings of lung IRI and the effects of SPMs in other lung injury models, SPMs should be produced in lung IRI and exert effects; however, only a few studies have investigated SPMs in lung IRI.[41–43] Although RvD1 has been reported to improve lung IRI,[42,43] few reports have clarified the detailed mechanisms behind this improvement. In addition, no study has examined the dynamic changes in endogenous SPMs and their precursors produced in vivo during the onset and resolution of lung IRI.

We showed that endogenous SPMs tended to be lower after reperfusion compared with naïve lungs. This is the first study investigating dynamic changes in endogenous SPMs during the initiation and resolution phases of lung IRI. In the initiation phase, it was suggested that the period of low endogenous SPMs was associated with deteriorating lung function. Intriguingly, the endogenous levels of SPMs during the resolution phase of lung IRI remained low for 7 days after reperfusion when oxygenation was improved. This observation was associated with the recovery of 17-HDHA and 15-HETE, the precursors of RvD1 and LXA4. It has been reported that the recovered level of endogenous RvD1 was associated with the improvement in lung injury in a rat model of LPS-induced acute lung injury.[44] Endogenous LXA4 was reported to be increased when lung injury was most

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severe and decreased during the recovery phase of the injury in a murine acid-induced lung injury model.[45] It has also been reported that endogenous LXA_4 was increased in the chronic phase of human lung transplantation.[46] Although the reason behind the differences in the dynamic changes of endogenous SPMs between lung IRI and other acute lung injury models was not identified in this study, it could be partly because the severity of lung damage was different among acute lung injury models or lung IRI was still in the process of recovery even after 7 days, as suggested by the relatively wide range of pO_2, insufficient improvement in airway pressure and compliance, and the recovery of resolvin and lipoxin precursors. Further studies are required to elucidate the underlying mechanisms of this difference.

Several studies have shown that SPMs exert their effects via G protein-coupled receptors, such as ALX/FPR2, GPR32, GPR18, and ChemR23.[19–22] In this study, we showed that among the SPM receptors ALX/FPR2, GPR18, and ChemR23, only ALX/FPR2 was upregulated in the IRI lung. ALX/FPR2 mRNA levels did not increase during WI; however, they increased after reperfusion. In addition, ALX/FPR2 was positive for inflammatory cells in the IRI lung. This suggests that the upregulation of ALX/FPR2 is mainly due to inflammatory cells infiltrating the IRI lung. The expression of ALX/FPR2 in other models of acute lung injury has been reported to be expressed in the airway epithelium and macrophages in a mouse model of pneumococcal pneumonia and acid-induced lung injury.[36,37] Absence of ALX/FPR2 increases susceptibility to pneumococcal pneumonia and displays uncontrolled inflammation in mice.[47] In addition, in IRI of other organs, ALX/FPR2 has been reported to be protective against damage.[48–51] Considering these reports, the findings from the current study may have potential

applications not only for the treatment of PGD but also for the clinical treatment of various lung injuries, such as pneumonia, and IRI of other organs.

To test the effect of SPM supplementation in the initiation phase of lung IRI, we focused on the SPMs agonistic to ALX/FPR2, namely RvD1 and LXA_4. RvD1 and LXA_4 are rapidly inactivated in vivo; however, their epimers used in this study, including AT-RvD1 and AT-LXA_4, have been reported to be relatively resistant to inactivation.[28–30] Therefore, we predicted that supplementation with more stable AT-RvD1 or AT-LXA_4 in the post-reperfusion period, when endogenous RvD1 and LXA_4 were reduced, could alleviate lung IRI. Our findings revealed that supplementation with AT-RvD1 and AT-LXA_4 improved various pulmonary physiological measurements. IL-1β and IL-6 levels were decreased in lung tissues. TNF-α was also reduced; however, statistical significance was achieved only with AT-LXA_4 administration. IL-1β, IL-6, and TNF-α are produced by multiple cells, such as macrophages, neutrophils, and lymphocytes, and have been reported to contribute to the development of lung IRI.[8,52] Neutrophil infiltration into lung tissue is also responsible for the induction of IRI and subsequent lung allograft rejection,[8,40,53,54] and we found that neutrophil infiltration was alleviated by AT-RvD1 and AT-LXA_4 administration. Additionally, lung edema and alveolar hemorrhage were also alleviated. Moreover, we found that the beneficial effects of AT-RvD1 and AT-LXA_4 on lung function and edema in lung IRI were not observed with the administration of WRW4 prior to AT-RvD1 and AT-LXA_4. WRW4 is a selective antagonist of ALX/FPR2 and inhibits the activation of ALX/FPR2.[55] Collectively, the protective effects of AT-RvD1 and AT-LXA_4 via ALX/FPR2 to prevent acute lung injury during the initiation phase of lung IRI were confirmed in this model.

The limitations of this study are as follows: First, in this study, we could not find a clear indication that endogenous SPMs were responsible for the resolution of lung IRI. The difference in the dynamic changes of endogenous SPMs between lung IRI and other acute lung injury models has not been identified. Second, we performed an observational study for the expression of ALX/FPR2 and the effects of AT-RvD1 and AT-LXA$_4$ in a rat lung IRI model, and we did not investigate the cellular mechanism of these effects. Immunohistochemical staining showed that inflammatory cells and airway epithelium expressed ALX/FPR2, and they could play important roles; however, this study did not elucidate the detailed cellular mechanisms. In addition, the effects of SPMs other than AT-RvD1 and AT-LXA$_4$ in lung IRI were not evaluated. Third, we could not verify whether AT-RvD1 and AT-LXA$_4$ would affect long-term graft survival after lung transplantation. The effects of AT-RvD1 and AT-LXA$_4$ diminished with longer reperfusion time, i.e., 3 h to 7 days after reperfusion, in our rat hilar clamp model. We selected this model for the study because it is a simpler, reliable, and more reproducible technique than the lung transplantation model. In this study, reperfusion was performed after WI. In contrast, in lung transplantation, the graft undergoes a period of cold ischemia followed by WI and reperfusion. Further investigations using a lung transplant model are required to address this issue.

In conclusion, in the rat hilar clamp model, endogenous SPMs in the lung tissue decreased immediately after reperfusion and remained low after the recovery of lung function. ALX/FPR2 was the only upregulated SPM receptor in the initiation phase of lung IRI, and administration of AT-RvD1 or AT-LXA$_4$ prevented exacerbation of lung IRI

via ALX/FPR2, which could contribute to the development of a new prophylactic or therapeutic strategy for IRI.

Acknowledgments

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Figure legends

Figure 1

Dynamics of lung function and wet to dry weight (W/D) ratio within 7days after lung ischemia-reperfusion injury

(A) Protocol for the reperfusion group.

(B) Peak airway pressure, (C) mean airway pressure, (D) dynamic, compliance, (E) oxygenation, and (F) W/D ratio in each group (n = 4/group).

*P < 0.05, **P < 0.01, ***P < 0.001. i.v., intravenous injection; R, reperfusion; h, hour or hours.

Figure 2

Dynamics of endogenous specialized pro-resolving lipid mediators (SPMs) after lung ischemia-reperfusion injury

Concentration of SPMs: (A) Resolvin (Rv) D1, (B) Pritectin D1, (C) Maresin 1, (D) Maresin 2, (E) RvE3, (F) Lipoxin (LX) A_4, (G) LXB_4, (H) 17-hydroxy docosahexaenoic acid (17-HDHA), and (I) 15-hydroxy eicosatetraenoic acid (15-HETE) in the left lung tissues (n = 4/group). *P < 0.05, **P < 0.01. R, reperfusion; h, hour or hours.

Representative tandem mass spectrometry fragmentation patterns and diagnostic ions (insets) employed for identification of (J) RvD1 and (K) LXA_4.

Figure 3

Expression and localization of specialized pro-resolving lipid mediator receptors after lung ischemia-reperfusion injury

(A) The protocols for warm ischemia (WI) and WI + reperfusion (WI + R) groups. Gene expression of (B) Formyl peptide receptor type 2 (ALX/FPR2) mRNA, (C) G protein-coupled receptor 18 (GPR18) mRNA, and (D) Chemerin receptor 23 (ChemR23) mRNA at different time points after reperfusion (n = 8/group).

(E) Immunohistochemical staining for ALX/FPR2 in the sham and WI + R groups. In the sham group, ALX/FPR2 was only expressed in the airway epithelium. In the WI + R group, ALX/FPR2 was expressed in inflammatory cells, in addition to the airway epithelium.

P < 0.05, **P* < 0.01, ***P* < 0.001. Arrowhead: representative ALX/FPR2 positive cells. Scale bars: 50 µm.

i.v., intravenous injection; Br, bronchus.

Figure 4

Aspirin-triggered (AT) resolvin D1 (AT-RvD1) or AT-lipoxin A_4 (AT-LXA$_4$) supplementation improves lung function after lung ischemia-reperfusion injury

(A) The protocols for vehicle and AT-RvD1 or AT-LXA$_4$ groups. (B) Peak airway pressure, (C) mean airway pressure, (D) dynamic, compliance, (E) oxygenation, and (F) wet to dry

weight ratio in each group (n = 8/group). The results of the sham group are indicated for reference. **$P < 0.01$, ***$P < 0.001$.

Figure 5

Aspirin-triggered (AT) resolvin D1 (AT-RvD1) or AT-lipoxin A_4 (AT-LXA$_4$) supplementation improves cytokine levels, lung edema, alveolar hemorrhage, and neutrophil infiltration after lung ischemia-reperfusion injury

The concentrations of cytokines (A) interleukin (IL)-1β, (B) IL-6, and (C) tumor necrosis factor (TNF)-α in the left lung tissue were evaluated using enzyme-linked immunoassay (n = 8/group). (D) Hematoxylin and eosin staining of the left lung of the sham, vehicle, AT-RvD1, and AT-LXA$_4$ groups. (E) Immunohistochemical staining for myeloperoxidase (MPO) in the left lung of the sham, vehicle, AT-RvD1, and AT-LXA$_4$ groups. (F) The index of the perivascular cuff area to the vessel area was calculated for five vessels per rat. (G) Average number of red blood cells in the alveolar space at five randomly chosen high-power fields (HPFs) per rat. (H) Average number of neutrophils at five randomly chosen HPFs per rat. The results of the sham group are indicated for reference. *$P < 0.05$, ***$P < 0.001$. Scale bars: 50 μm.

Figure 6

Formyl peptide receptor type 2 antagonist eliminates the effects of aspirin-triggered (AT) resolvin D1 (AT-RvD1) or AT-lipoxin A_4 (AT-LXA$_4$) on lung ischemia-reperfusion injury

(A) The protocols for WRW4 + vehicle and WRW4 + AT-RvD1 or AT-LXA$_4$ groups. (B) Peak airway pressure, (C) mean airway pressure, (D) dynamic, compliance, (E) oxygenation, and (F) wet to dry weight ratio in each group (n = 8/group). (G) Hematoxylin and eosin staining of the left lung in each group. Scale bars: 50 μm.
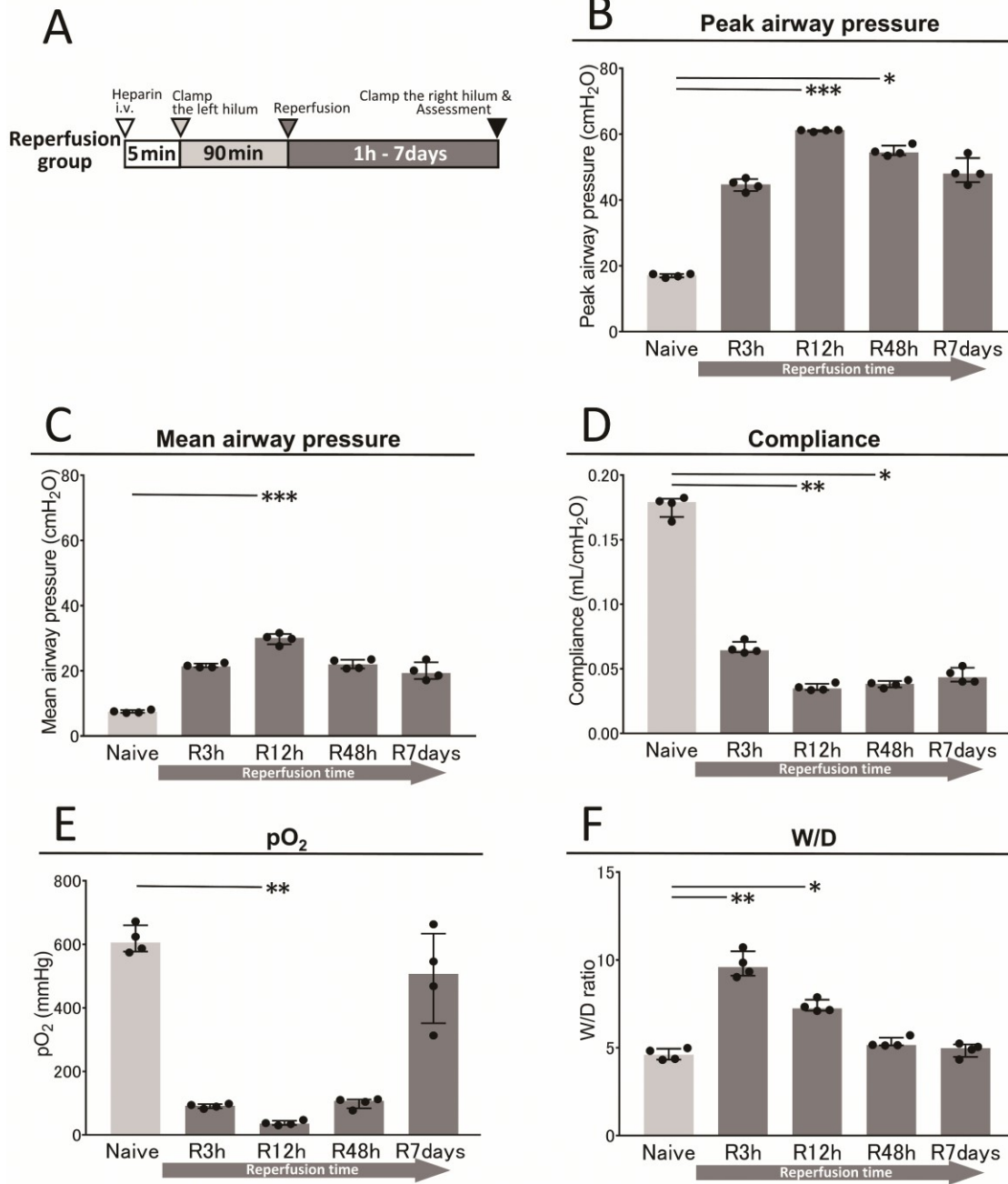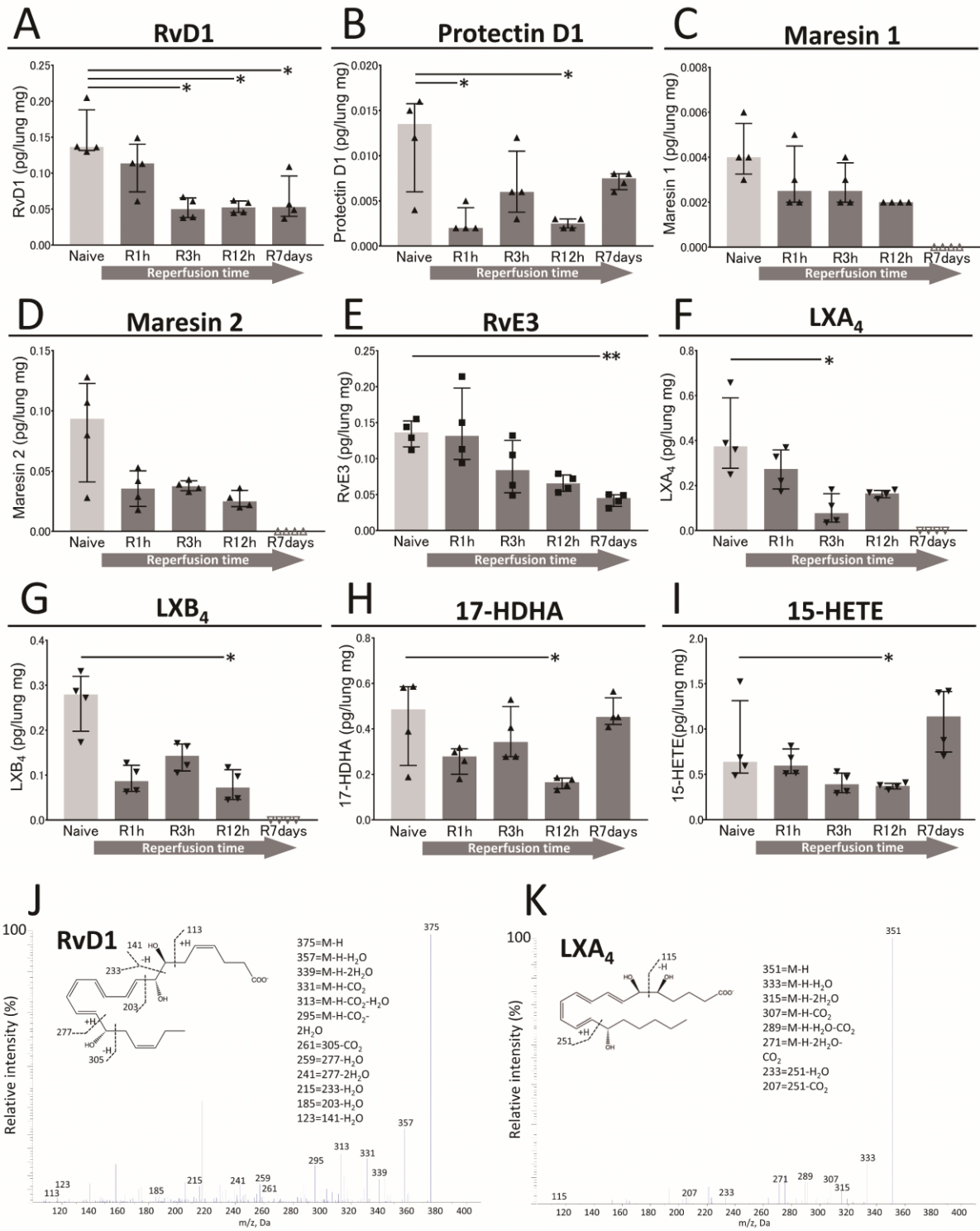
Figure 1

Figure 2

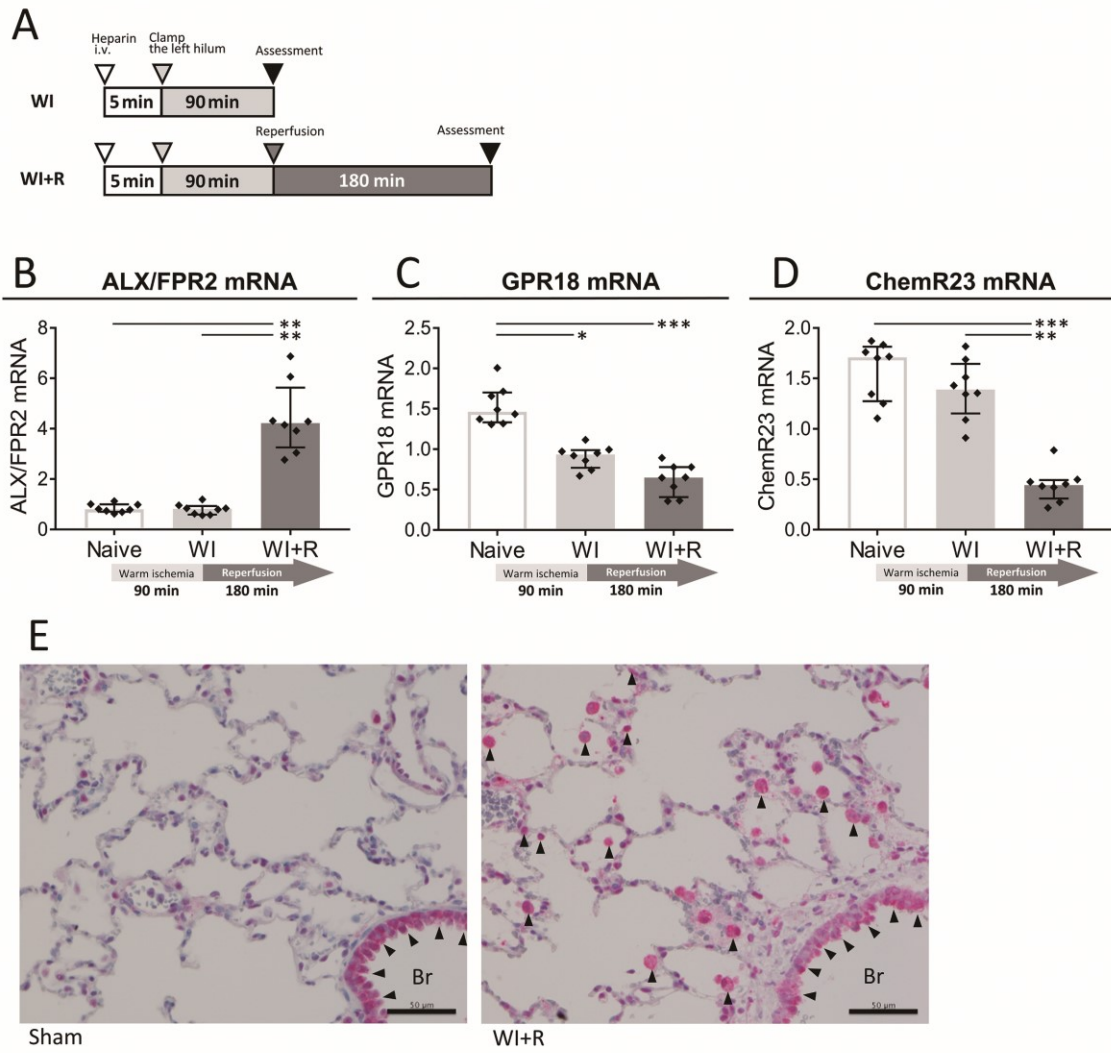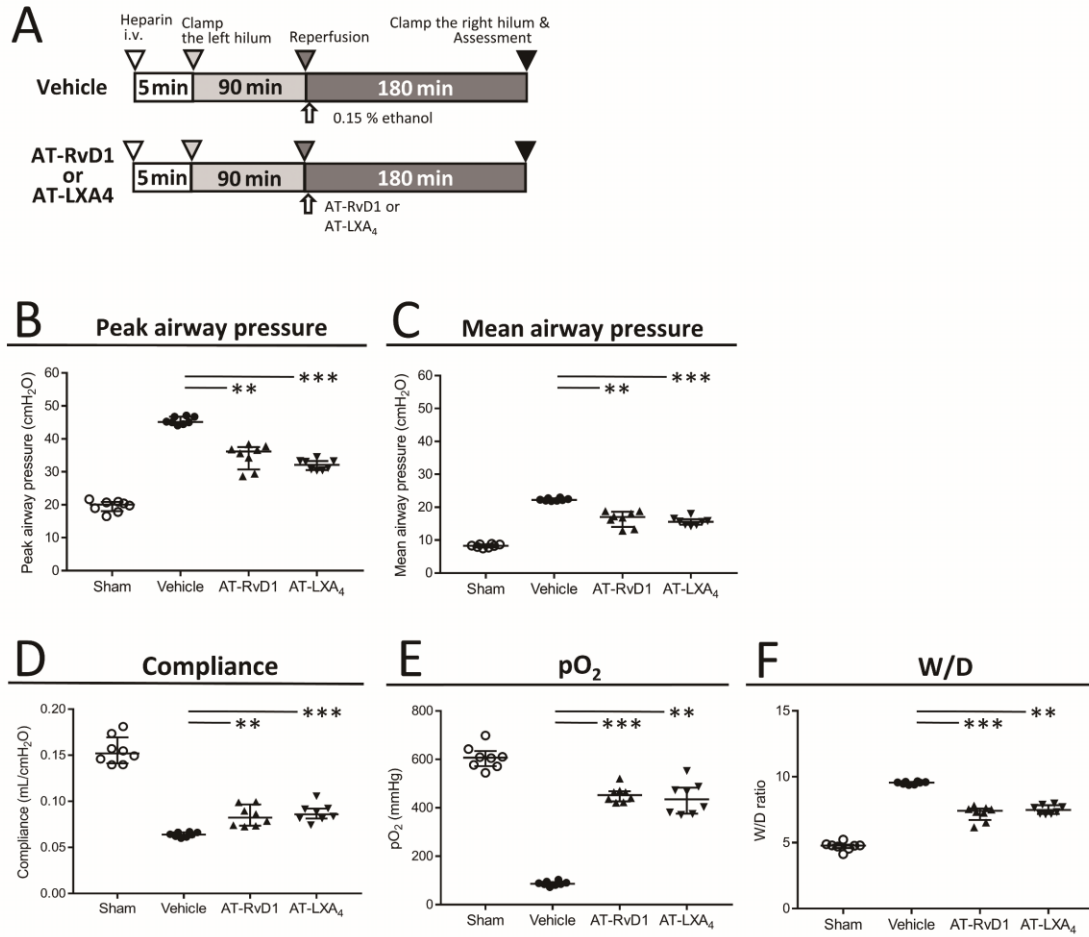**Figure 3**

Figure 4

Figure 5

Figure 6

WRW4 + Vehicle

Heparin i.v. | Clamp the left hilum | Reperfusion | Clamp the right hilum & Assessment

5 min | 90 min | 180 min

WRW4 ⇈ 0.15 % ethanol

WRW4 + AT-RvD1 or AT-LXA$_4$

5 min | 90 min | 180 min

WRW4 ⇈ AT-RvD1 or AT-LXA$_4$

B Peak airway pressure

Peak airway pressure (cmH$_2$O)

WRW4 + Vehicle | WRW4 + AT-RvD1 | WRW4 + AT-LXA$_4$

C Mean airway pressure

Mean airway pressure (cmH$_2$O)

WRW4 + Vehicle | WRW4 + AT-RvD1 | WRW4 + AT-LXA$_4$

D Compliance

Compliance (mL/cmH$_2$O)

WRW4 + Vehicle | WRW4 + AT-RvD1 | WRW4 + AT-LXA$_4$

E pO$_2$

pO$_2$ (mmHg)

WRW4 + Vehicle | WRW4 + AT-RvD1 | WRW4 + AT-LXA$_4$

F W/D

W/D ratio

WRW4 + Vehicle | WRW4 + AT-RvD1 | WRW4 + AT-LXA$_4$

G

WRW4+Vehicle | WRW4+AT-RvD1 | WRW4+AT-LXA$_4$