Intralipid Prevents and Rescues Fatal Pulmonary Arterial Hypertension and Right Ventricular Failure in Rats

Soban Umar, Rangarajan D. Nadadur, Jingyuan Li, Federica Maltese, Parisa Partownavid, Arnoud van der Laarse, Mansoureh Eghbali

Abstract—Pulmonary arterial hypertension (PAH) is characterized by pulmonary vascular remodeling leading to right ventricular (RV) hypertrophy and failure. Intralipid (ILP), a source of parenteral nutrition for patients, contains γ-linolenic acid and soy-derived phytoestrogens that are protective for lungs and heart. We, therefore, investigated the therapeutic potential of ILP in preventing and rescuing monocrotaline-induced PAH and RV dysfunction. PAH was induced in male rats with monocrotaline (60 mg/kg). Rats then received daily ILP (1 mL of 20% ILP per day IP) from day 1 to day 30 for prevention protocol or from day 21 to day 30 for rescue protocol. Other monocrotaline-injected rats were left untreated to develop severe PAH by day 21 or RV failure by approximately day 30. Saline or ILP-treated rats served as controls. Significant increase in RV pressure and decrease in RV ejection fraction in the RV failure group resulted in high mortality. Therapy with ILP resulted in 100% survival and prevented PAH-induced RV failure by preserving RV pressure and RV ejection fraction and preventing RV hypertrophy and lung remodeling. In preexisting severe PAH, ILP attenuated most lung and RV abnormalities. The beneficial effects of ILP in PAH seem to result from the interplay of various factors, among which preservation and/or stimulation of angiogenesis, suppression and/or reversal of inflammation, fibrosis and hypertrophy, in both lung and RV, appear to be major contributors. In conclusion, ILP not only prevents the development of PAH and RV failure but also rescues preexisting severe PAH. (Hypertension. 2011;58:512-518.) ● Online Data Supplement

Key Words: pulmonary arterial hypertension ■ Intralipid ■ inflammation ■ angiogenesis ■ hypertrophy

Category I pulmonary hypertension, also known as pulmonary arterial hypertension (PAH), includes idiopathic, familial, and acquired PAH. PAH is characterized by arterial obstruction resulting from excessive proliferation of pulmonary artery smooth muscle cells and endothelial cells, endothelial dysfunction, and inflammation. Progressive increase in pulmonary artery pressure eventually leads to right ventricular (RV) hypertrophy, RV failure (RVF), and sudden cardiac death. Currently available therapies for PAH only improve the symptoms and delay the progression of the disease. A definitive therapy for PAH is still lacking. Lipids and, in particular, polyunsaturated fatty acids, have recently received special cardiovascular research attention. Intralipid (ILP) is the first safe emulsion of fatty acids for human use. ILP has been widely used as a source of parenteral nutrition for patients for >4 decades. ILP 20% is an emulsion of soybean oil (20.0%), egg-yolk phospholipids (1.2%), and glycerol (2.2%). γ-Linolenic acid (GLA), one of the key essential fatty acids in ILP, has been shown to be protective against doxorubicin-induced cardiotoxicity. A soy-derived phytoestrogen, genistein, has been shown to ameliorate monocrotaline (MCT)-induced PAH. Because some of the constituents of ILP, like GLA and soy-derived phytoestrogens, are protective for lungs and heart, we hypothesized that ILP may prevent and rescue the development of PAH and RVF. We report that ILP prevents MCT-induced pulmonary and cardiac dysfunction by preserving both lung and RV structure. Most importantly, ILP is effective in rescuing severe preexisting PAH by restoring lung and RV structure and function.

Methods

For full methodological details, please see the online Data Supplement at http://hyper.ahajournals.org.

Animals and Treatments

Male Sprague-Dawley rats ~3 to 4 months old (350 to 400 g) were treated with a single SC dose (60 mg/kg) of MCT at day 0 to induce PAH by day 21 and RVF by day 30. Some MCT-treated rats received daily ILP (1 mL of 20% ILP per day IP) from day 1 to day 30 for prevention protocol or from day 21 to day 30 for rescue protocol. Rats treated with a single dose of PBS or daily injection of ILP for 30 days served as controls (CTRLs; Figure S1A and S1B, available online).
Cardiac and Pulmonary Hemodynamics
B-mode, M-mode, and pulmonary pulsed-wave Doppler echocardiography were performed (VisualSonics Vevo 770, 30-MHz linear transducer) to accurately monitor the stage of disease. Direct cardiac catheterization was performed to record RV pressure just before euthanization.6

Gross Histological Evaluation
The lungs, RV wall, left ventricular (LV) wall, and interventricular septum (IVS) were dissected. The wet lung weight and RV hypertrophy index [RV/(LV/IVS)] were determined.

Real-Time PCR
Total RNA from lungs and RV were isolated using TRIzol (Invitrogen) and reverse transcribed with oligo-dt using the Omniscript RT kit (Qiagen). CTRLs were the reaction without reverse transcriptase and H2O instead of cDNA.

Western Blot Analysis, Immunohistochemistry, and Imaging
Standard Western blot analysis was performed using whole lung and RV lysates. Tissue sections of hearts and lungs were stained with immunofluorescence, immunoperoxidase, standard hematoxylin-eosin, or Masson trichrome staining. The images were acquired using light microscopes (Zeiss Axiovert 135 and Nikon Eclipse E400) or with a high-resolution laser scanning confocal microscope (Olympus).

Statistical Analysis
One-way ANOVA tests were used to compare between groups using SPSS13.0 for Windows. When significant differences were detected, individual mean values were compared by post hoc tests (Bonferroni) that allowed for multiple comparisons. P<0.05 was considered statistically significant. Values are expressed as mean±SE.

Results
Improvement of Cardiopulmonary Function With ILP Therapy
A single injection of MCT induced RVF secondary to PAH in ~30 days (Figure S1). Peak systolic RV pressure increased sharply to 68±1 mm Hg at day 21 and further to 70±2 mm Hg at approximately day 30 (Figure 1A). RV ejection fraction was initially increased to 72±2% at day 14, a stage known as “compensated hypertrophy,” but later declined rapidly to 39±1% at day 21 and further to 31±3% at day 30, a clear indication of RVF (Figure 1B). ILP also prevented the sharp increase in RV pressure and the development of RVF by maintaining RV ejection fraction ~50% throughout the course of therapy (Figure 1A and 1B). Both RV pressure (34±2 in ILP versus 30±2 mm Hg in control [CTRL]) and RV ejection fraction (62.8±1.5% in ILP versus 66.0±1.0% in CTRL) did not differ significantly between ILP and CTRL at day 30. In the RVF group, mortality started as early as day 24 and reached 100% by day 32 (Figure S2). Daily ILP therapy from day 1 to day 30 resulted in 100% survival of rats and significantly improved body weight gain (Figure S2).

Because PAH is not always diagnosed early, we explored whether ILP could also rescue preexisting severe PAH. Because severe PAH was already evident at day 21 (Figures 1 and 2), we started ILP therapy at day 21 until day 30 (Figure S1). We found that even 10 days of ILP therapy was sufficient to rescue preexisting severe PAH, because RV pressure (43.9±1.1 mm Hg) and RV ejection fraction (53.6±0.4%) were rescued to a great extent (Figure 1C and
Figure 2. Intralipid (ILP) preserves and reverses the cardiopulmonary abnormalities induced by pulmonary arterial hypertension (PAH). A, Immunofluorescence labeling of pulmonary arterioles stained with anti-α-smooth muscle actin antibody (green). B, Hematoxylin-eosin staining of heart cross-sections. C, Echocardiographic images of pulse-waved Doppler (taken at day 30 except for PAH group at day 21) show midsystolic notch (arrows) present in pulmonary artery (PA) flow in PAH and right ventricular (RV) failure (RVF) groups only. Masson trichrome staining of lung (D) and RV sections (E), arrows point to fibrosis (blue). Quantification of pulmonary arteriolar medial wall thickness (F), lung fibrosis (G), and RV fibrosis (H), please see the online Data Supplement at http://hyper.ahajournals.org for quantification details: **P<0.01 vs control (CTRL); ***P<0.001 vs CTRL; $$$P<0.001 vs PAH; ##P<0.01 vs RVF; ###P<0.001 vs RVF; *P<0.05 vs ILP (n=3 to 4 animals per group). Res indicates rescue.
1D). These data suggest that ILP not only prevents PAH but also rescues preexisting severe PAH.

Preservation and Reversal of Cardiopulmonary Structure With ILP Therapy
PAH also led to structural changes in the lungs and RV. ILP prevented the increase in lung weight (1.80±0.06 g in ILP versus 2.38±0.20 g in RVF; 1.36±0.10 g in CTRL; Figure S1C), pulmonary arteriolar medial hypertrophy (Figure 2A and 2F), RV hypertrophy (RV/[LV+IVS]=0.33±0.04 in ILP versus 0.68±0.10 in RVF; 0.24±0.06 in CTRL; Figure S1D), RV dilatation (Figure 2B), and the increase in RV end-diastolic diameter (Figure S2A). ILP was also able to reverse preexisting severe lung and RV remodeling (lung weight: 2.01±0.05 g in ILP-rescue (Res) versus 2.39±0.15 g in PAH; RV hypertrophy: 0.39±0.02 in ILP-Res versus 0.65±0.08 in PAH; Figure S1C and S1D). ILP also prevented and reversed both the increase in pulmonary artery (PA) acceleration time and appearance of midsystolic notching on pulmonary artery flow (Figure 2C).

ILP Ameliorates and/or Reverses Cardiopulmonary Fibrosis and Inflammation Associated With PAH-Induced RVF
ILP therapy was associated with significantly lower total fibrosis in the lungs (4.46±0.92% in ILP versus 18.00±1.00% in RVF; 3.35±0.35% in CTRL; Figures 2D, 2G, and S3) and RV (5.53±0.67% in ILP versus 34.30±2.90% in RVF; 3.33±0.43% in CTRL; Figures 2E, 2H, and S3). Interestingly, ILP was also able to reverse the preexisting fibrosis in both lung (5.80±0.48% in ILP-Res versus 17.33±2.00% in PAH) and RV (7.99±1.05% in ILP-Res versus 21.30±0.66% in PAH).

In addition to fibrosis, ILP not only prevented but also reversed the significant increase in ED1-positive inflammatory cells in the PAH and RVF (cells per field: 19±3 in ILP versus 70±4 in RVF, 14±2 in CTRL; 28±3 in ILP-Res versus 64±2 in PAH; Figure 3A and 3B). In addition, in the prevention protocol, ILP therapy was associated with significantly lower levels of interleukin (IL) 6 transcript (one of the known proinflammatory markers of PAH; Figure 3C and 3D) and cleaved caspase 3 levels in both tissues (Figure S4). These data suggest that suppression and/or reversal of lung macrophages and cardiopulmonary fibrosis, as well as restoration of IL-6 and caspase 3 (in the prevention protocol) by ILP, could be the mechanisms contributing to improved cardiopulmonary function.

ILP Prevents and Rescues Loss of Capillaries in RV
We examined whether preservation or stimulation of cardiac capillary growth participated in ILP-induced prevention and/or rescue of PAH. ILP therapy not only prevented the loss of blood vessels observed in RVF (microvessels/cardio-myocytes normalized to CTRL: 1.03±0.001 in ILP versus 0.520±0.070 in RVF), it even stimulated capillary growth by fully restoring the loss of capillaries in RV (0.980±0.002 in ILP-Res versus 0.690±0.070 in PAH; Figure 4A and 4B). Concomitant with decline of capillary density, there was a significant reduction of proangiogenic vascular endothelial growth factor (VEGF) protein levels in lungs and RV of RVF, which was fully restored by ILP prevention therapy in both lungs and RV (Figure 4C and 4D).

Discussion
Here we show that ILP, the first clinically safe lipid emulsion for parenteral nutrition in humans, not only prevents the
progression of PAH to RVF but also effectively rescues preexisting severe PAH by reversing cardiopulmonary dysfunction in rats. ILP therapy resulted in 100% survival, whereas mortality in untreated rats reached 100% by day 32 (Figure S2). The complete disappearance of the characteristic midsystolic notching on pulmonary artery flow in PAH and RVF rats was another very strong indicator of the effective prevention and rescue action of PAH by ILP (Figure 2C). ILP was not only able to prevent the onset of PAH-induced RVF but also to reverse the deleterious effects of preexisting PAH. We demonstrated that preservation and/or stimulation of angiogenesis, suppression and/or reversal of hypertrophy, inflammation, and fibrosis are pivotal events associated with the therapeutic efficacy of ILP (Figure S5).

**ILP Therapy Preserves and Rescues Lung and Heart Structure and Function**

Our results clearly demonstrate that ILP prevents the development of fatal PAH and rescues even established preexisting PAH. Because ILP has been shown to protect the heart (against ischemia/reperfusion injury) and local anesthetic-induced cardiac arrest) and lungs (adult dogs and newborn rats), we speculate that the beneficial actions of ILP are mediated through its combined protective effects on the lungs and on the heart. One of the key essential fatty acids in ILP, GLA, is a precursor of prostacyclin, a potent pulmonary vasodilator and platelet aggregation inhibitor that is effective in the treatment of PAH. GLA improves lung microvascular permeability, oxygenation, and cardiopulmonary function and reduces proinflammatory eicosanoid synthesis and lung inflammation. Other than GLA, ILP also contains 20% soybean oil. Genistein, a soy-derived phytoestrogen also found in ILP, has been shown previously to ameliorate MCT-induced PAH. Thus, the combined effects of the soy and GLA, along with other components found in ILP, offer even more optimal and global cardiopulmonary benefits against PAH.

**ILP Ameliorates Lung and RV Inflammation, Fibrosis, and Apoptosis Associated With PAH**

We have observed severe lung and RV remodeling in PAH and RVF, as reported previously, as well as pulmonary and...
RV fibrosis (Figures 1 and 2). ILP not only prevented but also reversed these cardiopulmonary abnormalities observed in PAH-induced RVF.

Inflammation plays an important role in the progression of PAH in animal models, as well as in PAH patients, most notably in the lungs and the RV. IL-6 is an established proinflammatory cytokine in PAH, and its serum levels are elevated in PAH patients. In fact, IL-6 serum levels could predict patient survival, because these levels were correlated with the severity of the disease. Here we show that ILP therapy prevented the upregulation of IL-6 both in the lungs and the RV of PAH rats. Furthermore, ILP not only prevented but also rescued the accumulation of macrophages/monocytes in the lungs (Figure 3). RV cardiomyocyte apoptosis has been shown recently to be a key mechanism in the progression of RVF secondary to PAH. The significant upregulation of IL-6 shown previously to be a key mechanism in the progression of PAH-induced RVF.

Preservation and/or Stimulation of Cardiopulmonary Angiogenesis by ILP

RV ischemia has been described in the hearts of PAH patients with normal coronary arteries caused by increased oxygen demand and loss of RV microvessels. Stimulation of pulmonary neoangiogenesis has been suggested as treatment of PAH-induced RVF. Cardiac angiogenesis has also been shown to be a key event in maintaining heart function during adaptive hypertrophy. We demonstrated that ILP therapy not only preserved the capillary density but also stimulated capillary growth and regeneration in the RV after the onset of PAH. The decrease in RV capillary density has been proposed to be caused by an insufficient upregulation of VEGF. We found a significant reduction of VEGF protein in lungs and RV of RVF. ILP was able to prevent VEGF downregulation in both tissues (Figure 4C and 4D), which may also contribute to its beneficial effect. Therefore, increased myocardial blood vessels and preservation of VEGF by ILP both in lung and heart may underlie the prevention of RVF and decrease in PH severity, thus leading to marked improvements in cardiopulmonary structure and function.

Conclusion

In conclusion, ILP prevents and rescues the development of MCT-induced PAH, RV hypertrophy, and RVF by preserving and even reversing the abnormalities in lung and heart structure and function. Suppression and/or reversal of inflammation, fibrosis, and hypertrophy, as well as preservation and/or stimulation of angiogenesis, are vital mechanisms in ILP-induced prevention and rescue of PAH (Figure S5). Our findings raise the exciting prospect that ILP, a clinically safe lipid emulsion for human use, may be used for the prevention/treatment of PAH and consequent RVF, which most definitely warrants further investigation.

Perspectives

Despite significant advances in cardiopulmonary research, PAH still remains a difficult disease to treat, because therapeutic strategies to simultaneously reduce pulmonary vascular damage and prevent RV dysfunction are lacking. The hallmark of PAH is remodeling of pulmonary vasculature, resulting in RV hypertrophy, and failure. Because daily administration of ILP protected rats from MCT-induced PAH, attenuated RV dysfunction, and proved to be very effective in rescuing severe preexisting PAH, ILP promises new clinical applications in patients with PAH.

Sources of Funding

This work was supported by grants HL089876, HL089876S1, and HL088640 (to M.E.).

Disclosures

None.

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Hypertension. 2011;58:512-518; originally published online July 11, 2011; doi: 10.1161/HYPERTENSIONAHA.110.168781

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INTRALIPID PREVENTS AND RESCUES FATAL PULMONARY ARTERIAL HYPERTENSION AND RIGHT VENTRICULAR FAILURE IN RATS

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Online data supplement

Methods

Animals and treatments. Male Sprague-Dawley rats (3-4 month old, 350-400 g) were used. To induce PAH, rats were treated with a single subcutaneous injection of MCT (60 mg/kg, Sigma) at day-0. MCT was dissolved in 1N HCl, the pH was adjusted to 7.4, and diluted with PBS before injection. Two protocols were used.

Prevention protocol:
One group of MCT injected rats received daily intraperitoneal ILP therapy from day 1 to day 30 (ILP group, 1mL of 20% ILP/day, n=8, Fig. S1A). We designed our experiment to start the first treatment of ILP at least 24h after injection of MCT to allow enough time for MCT to get metabolized in the liver. In fact it has been shown by Wilson et al. and others that this 24h period is not only sufficient for the hepatic conversion of MCT into its active metabolite and its plasma clearance to occur, but also for its binding to the pulmonary vascular endothelium of smaller vessels to initiate pulmonary vascular lesions leading to PAH\textsuperscript{1,2}. The other MCT group was left untreated to develop RV failure (RVF group) by day 30 (n=13). PBS, or daily injection of ILP for 30 days served as controls (Fig. S1A).

Rescue protocol:
One group of MCT rats was sacrificed 21 after MCT when PAH had already been established\textsuperscript{3,4} (PAH group, n=7), whereas another MCT treated group was given daily intraperitoneal injections of ILP for 10 days starting at day 21 (ILP-Res group, n=7, Fig. S1B).

Animals in both groups were monitored with serial echocardiography and regular body weight measurements. A weight loss of more than 10% per day for 2 days and arterial oxygen tension of less than 80% were the criteria required to sacrifice the animals and counted as a ‘loss’ in survival measurements. Protocols received institutional review and committee approval.

Cardiac and pulmonary hemodynamics. Rats were anesthetized with inhaled isoflurane (4% for induction and 2% for maintenance) for echocardiography. Serial pulsed-wave Doppler echocardiography of pulmonary artery (PA) flow was performed during the course of experiment at day 0, 14, 21, 25, 27 and 30 (in all groups except in PAH that echocardiography was performed on day 0, 14 and 21 as these animals were sacrificed at day 21) to accurately monitor the progression of the disease. The RV pressure was calculated using Mahan’s regression equation: MPAP= 79 – (0.45×PAAT)\textsuperscript{5}, in which MPAP is mean pulmonary artery pressure and PAAT is the pulmonary artery acceleration time. The RV pressure was also measured directly by inserting a catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, ADInstruments) into the RV right before sacrifice. For cardiac catheterisation, the rats were anesthetized with a mixture of Ketamine (80 mg/kg) and Xylazine (8 mg/kg) intraperitoneally. The animals were placed on a controlled warming pad to keep the body temperature constant at 37°C. After a tracheotomy was performed, a cannula (18G, Biovalve) was inserted, and the animals were mechanically ventilated. After a midsternal thoracotomy, rats were placed under a stereomicroscope (Zeiss, Hamburg, Germany) and a pressure-conductance catheter (model 1.4F Millar SPR-671) was introduced via the apex into the RV and positioned towards the pulmonary valve. The catheter was connected to a signal processor (ADInstruments) and RV pressures were recorded digitally. After recording the
pressures, heart and lung tissues were removed rapidly under deep anaesthesia for preservation of protein integrity. The values of RV pressure measured by both methods (Echocardiography and direct RV catheterisation) were, on average, similar.

**Western Blot analysis.** RV and lungs were homogenized at 4°C in (mM): 150 NaCl, 50 Tris-HCl, 1 EGTA, 1 EDTA, 1 NaF, 1 PMSF, 1 Na3VO4, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate (pH 7.4) supplemented with protease and phosphatase inhibitor cocktails (Roche). The samples were centrifuged at 12,000 g for 10 min and the supernatants were collected. Protein concentration was measured and 100 μg of total protein was loaded on a 4-20% gradient Tris-HCl/SDS polyacrylamide gel, electrotransferred to nitrocellulose paper, blocked with 5% non-fat dry milk in 20 mM TBS with 0.1% Tween, and incubated with primary antibodies. Blots were then indirectly labelled using infrared fluorophore conjugated anti-rabbit and anti-mouse secondary antibodies for 1 h, and visualized with the Odyssey™ Imaging System (Li-Cor). Equal loading of protein onto each lane in the gel was confirmed by probing for vinculin or GAPDH. In the immunoblots, all samples from CTRL, RVF and ILP were run on the same gel or on two gels at the same time due to the lack of space. The blots were incubated together with the primary and secondary antibodies and were scanned together with the same laser intensity. Since we are only showing two representative lanes from a total of 3-5 samples per group, some of the intervening lanes were not shown and separated by a dotted line if the samples were run on the same gel, or a continuous line if they were from different blots.

**Immunocytochemistry and imaging.** Whole heart and lung were fixed in 4% paraformaldehyde (PFA) in 0.1 M Na2HPO4 and 23 mM NaHPO4 (pH 7.4) for 4 h on ice. The tissue was then immersed in ice-cold 20% sucrose in 0.1 M Na2HPO4 and 23 mM NaHPO4 (pH 7.4) overnight to cryoprotect the tissue, mounted using OCT and transversal 6-7 μm sections were obtained with a cryostat. Tissue sections were stained with immunofluorescence, immunoperoxidase, standard eosin/hematoxylin and Masson trichrome staining. The images were acquired using light microscopes (Axiovert 135, Zeiss, and Nikon Eclipse E 400) or with a laser scanning confocal microscope (Olympus).

- **Immunofluorescence staining.** Hearts cross-sections (6-7 μm) were fixed in acetone for 15 min at –20°C. The sections were then washed with PBS+0.1% Triton three times, incubated with 10% normal goat serum in PBS+0.1% Triton for 30 min to block the background. The sections were then incubated with primary antibodies in PBS+0.1% Triton+ 1% normal goat serum at 4°C overnight. The sections were then washed with PBS+0.1% Triton three times, incubated with the appropriate secondary antibodies in PBS+0.1% Triton+ 1% normal goat serum at room temperature for 1 h. After washing the secondary antibodies with PBS+0.1% Triton three times, the sections were incubated with wheat germ agglutinin (WGA, 1:200 dilution) in PBS+0.1% Triton+ 1% normal goat serum for 1 h at room temperature. The sections were then washed with PBS three times and mounted using Prolong gold (Molecular Probes) for imaging.

- **Immunoperoxidase staining.** Endogenous peroxidase activity was inhibited by incubating the lung sections with 0.3% H2O2 in PBS for 20 min at room temperature followed by washing with PBS three times. Lung sections were then incubated with 10% normal goat serum in PBS containing 0.1% Triton for 30 min at room temperature to
block the nonspecific binding. The sections were incubated with the appropriate primary antibodies in PBS+0.1% Triton+ 1% normal goat serum at 4°C overnight, washed 3 times with PBS+0.1% Triton. The sections were incubated with HRP-conjugated secondary antibody (1:200 dilution) in PBS+0.1% Triton+ 1% normal goat serum, for 1 h at room temperature, washed with PBS+0.1% Triton three times and stained with diaminobenzidine (DAB) metal substrate (10X DAB solution + stable buffer) for 5-10 min. The DAB was rinsed with H2O, stained with hematoxylin for 1 min, and washed under running tap water for 10 min. Dehydration was then performed by incubating in 50%, 70%, 96%, 100% ethanol and xylene. Sections were mounted using Permount (Fisher Scientific) and examined under a light microscope.

**Histological Analyses**

**Pulmonary arteriolar medial wall thickness**

Pulmonary arteriolar medial wall thickness was determined using α-smooth muscle actin staining of pulmonary arterioles in lung sections. The wall thickness of arterioles was quantified using ImageJ software by measuring the maximum thickness of arteriolar walls. The values are normalized to control (100%). There were at least 5-10 arterioles measured per slide and 2 slides per animal (n=3-4 rats per group). Sections were randomly selected and the observers were blinded.

**Percent tissue fibrosis**

Percent tissue fibrosis in lung and RV sections was determined using Masson trichrome staining for collagen with the use of a grid that divided the field of view into 100 squares. The number of collagenous tissue (blue stain) at the 100 intersection points in the grid was scored as 1 (present) or 0 (absent). Results are expressed as the percentage occupied by fibrosis to the total area examined. There were at least 5 fields of view recorded per slide at 20x magnification and 2 slides per animal (n=3-4 rats per group). Sections were randomly selected and the observers were blinded.

**ED1 positive macrophage/monocytes**

ED1 positive macrophage/monocytes in lung sections were quantified using ED1 immunostaining. Number of ED1 cells counted per 20x field from at least 5 fields per slide, 2 slides per animal (5 rats per group). Sections were randomly selected and the observers were blinded.

**RV capillary density**

RV capillary density in RV sections was quantified as the number of capillaries per cardiomyocytes using CD31 and wheat germ agglutinin immunostaining. Capillary density was quantified from at least 5 high power field (60x) per slide, 2 slides per animal (3 rats per group). Sections were randomly selected and the observers were blinded.

**Reagents.** Primary antibodies used were: anti-smooth muscle actin (Thermo Scientific, 1:200), anti-PECAM (CD31, Millipore, 1:200), anti-ED1 (CD68, Millipore, 1:200), anti-VEGF (Santa Cruz Biotechnology Inc., 1:200, this VEGF antibody recognizes the pro-angiogenic splice variants of VEGF; VEGF189, VEGF165 and VEGF121), anti-caspase-3 (Cell Signaling, 1:200), anti-vinculin (Sigma, 1:10,000) and anti-GAPDH (Novus Biologicals, 1:1000). Secondary antibodies used were goat anti-rabbit-IgG-AlexaFluor488 (1:1000) and goat anti-mouse-IgG-AlexaFluor568 (1:1000) for
immunofluorescence, sheep anti-mouse-IgG-HRP (1:200), and sheep anti-rabbit-IgG-HRP (1:200) for immunoperoxidase stainings, goat anti-rabbit-IgG-AlexaFluor680 (1:100,000, Invitrogen) and goat anti-mouse-IgG-IRDye800CW (1:100,000, Odyssey, LI-COR) for western immunoblotting.

References


Figure S1. ILP prevents and rescues severe PAH. 

A. Prevention protocol. Male rats were treated with a single subcutaneous injection of MCT (60 mg/kg) at day-0 to develop RVF by day 30 (n=13). One group of MCT injected rats received daily intraperitoneal ILP therapy from day 1 to day 30 (ILP group, 1mL of 20% ILP/day, n=8). PBS (n=7), or daily injection of ILP for 30 days served as controls (n=3). 

B. One group of MCT rats was sacrificed 21 after MCT when PAH had already been established (PAH group, n=7), whereas another MCT treated group was given daily intraperitoneal injections of ILP for 10 days starting at day 21 until day 30 (ILP-Res group, n=7). PBS treated rats served as controls. 

C. Lung weight and D. RV hypertrophy index (RV/LV+IVS) in PBS control and ILP control (CTRL, black bars), PAH (purple bar), RVF (red bar), ILP (blue bar) and ILP-Res (green bar). *P<0.05 vs. CTRL; ***P<0.001 vs. CTRL; #P<0.05 vs. RVF; ###P<0.001 vs. RVF; $P<0.05 vs. PAH; $$P<0.01 vs. PAH; $$$P<0.001 vs. PAH; ^P<0.05 vs. ILP-CTRL; ^^P<0.01 vs. ILP-CTRL; ^^^P<0.001 vs. ILP-CTRL (n=7-8 rats per group, except ILP-CTRL n=3).
Figure S2. Preservation of cardiopulmonary function, improved survival and weight gain with ILP therapy. A. Echocardiographic images of M-mode of heart, where RV is right ventricle, LV is left ventricle, IVS is inter-ventricular septum and EDD is end-diastolic diameter. B,C. Averaged RV pressure (RVP) and RV ejection fraction (RVEF) in PBS CTRL and ILP CTRL (black bars), PAH (purple gray), RVF (red bar), ILP (blue bar) and ILP rescue (green bar) at the end of experiment at day 30, except for PAH group at day 21. **P<0.01 vs. CTRL; ***P<0.001 vs. CTRL; $$$P<0.001 vs. PAH; ####P<0.001 vs. RVF; ^P<0.05 vs. ILP; ^^P<0.01 vs. ILP; @@P<0.01 vs. ILP-CTRL; @@@P<0.001 vs. ILP-CTRL (n=3-8 animals per group). D. Survival plot of ILP treated (blue squares) and untreated rats (red circles). E. Body weight gain in CTRL (black bar), RVF (red bar) and ILP (blue bar) during 30 days of experiment. **P<0.01 vs. CTRL; #P<0.05 vs. RVF (n=7-8 animals per group).
Figure S3. Suppression of cardiopulmonary fibrosis by ILP. Masson trichrome staining showing high-magnification images of lung (A) and RV sections (B) from CTRL, PAH, RVF, ILP and ILP-Res groups for fibrosis (blue).
**Figure S4. Suppression of cardiopulmonary apoptosis by ILP.** Representative immunoblots of lung (A) and RV (B) lysates from CTRL, RVF and ILP labeled with anti-caspase-3 and anti-vinculin antibodies. All samples from CTRL, RVF and ILP were run on the same gel. Since we are only showing two representative lanes from a total of 3-4 samples per group, some of the intervening lanes were not shown and separated by a dotted line. Western blot analyses of cleaved caspase-3 protein normalized to vinculin in CTRL (black bar), RVF (red) and ILP (blue) are also shown. *P<0.05 vs. CTRL; **P<0.01 vs. CTRL; #P<0.05 vs. RVF (n=3-4 animals per group).
Figure S5. Proposed mechanisms underlying ILP-induced prevention and rescue of PAH and RVF. Injection of MCT induces PAH in healthy rats and eventually leads to RVF if left untreated. If ILP therapy started 1 day after MCT (prevention protocol), it prevents MCT-induced PAH and RVF possibly by stimulation of angiogenesis along with suppression of inflammation, hypertrophy, apoptosis and fibrosis. If ILP therapy is started 21 day after MCT when the severe PAH is already present (rescue protocol), it not only prevents the transition of PAH to RVF, but also rescues pre-existing PAH by reversing RVP, RVEF, RVH, lung inflammation, cardiopulmonary fibrosis and improves RV angiogenesis. RV, right ventricle; RVF, right ventricular failure; ILP, Intralipid®; RVEF, right ventricular ejection fraction; MCT, monocrotaline; PAH, pulmonary arterial hypertension; RVH, right ventricular hypertrophy; VEGF, vascular endothelial growth factor and IL-6, interleukin-6.