Salusin- β contributes to vascular inflammation associated with pulmonary arterial hypertension in rats

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ABSTRACT

Objective: Inflammation is closely linked to pulmonary arterial hypertension (PAH). Salusin- β , a bioactive peptide, has been reported to participate in vascular inflammation. We therefore hypothesized that salusin- β contributes to monocrotaline (MCT)-induced PAH in rats.

Methods: Male Sprague-Dawley rats were treated with MCT (60 mg kg⁻¹, single intraperitoneal injection). Salusin- β expression in the lungs of the MCT-treated rats was evaluated using immunofluorescence staining, western blot, and real-time PCR. For salusin- β blockade assay, rats injected with MCT were given a chronic infusion of anti-salusin- β immunoglobulin G (IgG) (salusin- β blocker, 1.0 μ g kg⁻¹ h⁻¹) or isotype-matched control IgG. Four weeks after MCT+anti-salusin- β treatment, the effects of salusin- β blockade were determined using hemodynamics, western blot, real-time PCR, and immunohistochemical detection. The effect of salusin- β on human pulmonary arterial endothelial cell (HPAEC) function was detected by adhesion and tube formation experiments in vitro.

Results: Salusin- β expression was significantly increased in the lungs of the MCT-treated rats, and immunofluorescence results showed that salusin- β was predominantly expressed in pulmonary macrophages and vascular endothelial cells. Salusin- β blockade significantly ameliorated PAH by acting against pulmonary vascular remodeling, decreasing macrophage infiltration, and reducing pro-inflammatory cytokine expression and nuclear factor-kappa B (NF- κ B) activity in the lungs of the MCT-treated rats. In addition, salusin- β could induce cell adhesion and accelerate angiogenesis by activating the NF- κ B pathway and promoting pro-inflammatory cytokine expression in the cultured HPAECs. This effect was suppressed by addition of the NF- κ B inhibitor, N-acetyl-L-cysteine.

Conclusions: Salusin- β plays a crucial role in the development of MCT-induced PAH models. (J Thorac Cardiovasc Surg 2016;152:1177-87)

The proposed model of salusin- β regulating PAH by activation of the NF- κ B pathway.

Central Message

Salusin- β contributes to monocrotaline-induced PAH through a pro-inflammatory effect.

Perspective

This study shows that salusin- β plays an important role in MCT-induced PAH in rats by activating the NF- κ B pathway. Inhibition of salusin- β expression ameliorates PAH development and right ventricular hypertrophy, which might be useful for treating patients with idiopathic PAH, PAH associated with systemic sclerosis disease, or infections.

See Editorial Commentary page 1188.

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Pulmonary arterial hypertension (PAH) is a life-threatening clinical syndrome characterized by pulmonary vascular remodeling, which results in a mean pulmonary artery pressure above 25 mm Hg and right ventricular failure.^{1,2} In recent years, large amounts of evidence from both animal models and human patients have suggested that inflammation plays a

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Abbreviations and Acronyms

α -SMA	= α -smooth muscle actin
HPAEC	= human pulmonary arterial endothelial cell
IgG	= immunoglobulin G
$\text{I}\kappa\text{B}\alpha$	= nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor, alpha
IL	= interleukin
MCT	= monocrotaline
MCP-1	= monocyte chemoattractant protein-1
PAH	= pulmonary arterial hypertension
$\text{NF-}\kappa\text{B}$	= nuclear factor kappa light chain enhancer of activated B
RVSP	= right ventricular systolic pressure
VCAM-1	= vascular cell adhesion molecular-1
vWF	= von Willebrand factor

key role in the pathogenesis of PAH.^{3,4} Perivascular inflammation has frequently been observed in patients with all forms of PAH, such as idiopathic PAH, drug- and toxin-induced PAH, and PAH associated with systemic autoimmune diseases.⁵ However, the molecular mechanisms that promote the remodeling and obstruction of pulmonary arterioles in patients with PAH are still not fully understood.

Salusin- β is a bioactive peptide consisting of 20 amino acids, which is translated from an alternatively spliced mRNA of the torsion dystonia-related gene TOR2A.⁶ Salusin- β has been found in human, rat, and mouse tissues, including the vasculature, the central nervous system, and body fluids, and plays a variety of roles in the cardiovascular, endocrine, and immune systems.^{7,8} Salusin- β has been reported to contribute to vascular remodeling associated with atherosclerosis via inflammatory responses in vascular endothelial cells, and to promote vascular smooth muscle cell proliferation.⁹ Salusin- β is also involved in the inflammatory processes associated with myocardial ischemic disease,¹⁰ hypertension,¹¹ and Behçet disease,¹² which indicates that salusin- β could affect the inflammatory processes associated with PAH.

Nuclear factor-kappa B (NF- κ B) is the key inflammatory transcription factor, which consists of p50, p52, c-Rel, RelB, and p65. Activation of NF- κ B expression has been reported in patients with idiopathic PAH and in rats with monocrotaline (MCT)-induced PAH,^{13,14} which further promotes the dysfunction of vascular endothelial cells and leads to the progression of vascular remodeling.¹⁵ Moreover, salusin- β directly induces inflammatory responses in vascular endothelial cells and causes the formation of macrophage foam cells by activating the NF- κ B pathway in coronary atherosclerosis.^{16,17} Here,

we hypothesized that salusin- β plays a role in the pathogenesis of MCT-induced PAH in rats by modulating the NF- κ B signaling pathway.

METHODS**Animal Models**

All animal care and experimental procedures were approved and conducted in accordance with the Institutional Animal Care and Use Committee of the Capital Medical University of China. Male Sprague-Dawley rats, weighing 220 to 250 g (Vital River Laboratories Animal Company, Beijing, China), were intraperitoneally (i.p.) injected with a single dose of MCT (60 mg kg⁻¹; Sigma-Aldrich, St. Louis, Mo) to induce severe pulmonary hypertension within 2 and 4 weeks; the controls were injected with an equal volume of 0.9% saline (0 weeks) (n = 8 each group). For salusin- β blockade studies, the MCT-injected rats were randomly divided into 3 groups: MCT (n = 12); MCT + isotype-matched control immunoglobulin G (IgG) (MCT + IgG) (n = 6); and MCT + anti-salusin- β IgG (n = 6). The MCT-treated rats were subcutaneously implanted with mini-osmotic pumps (model 2004; ALZET, Cupertino, Calif) and continuously infused with anti-salusin- β IgG (1.0 μ g kg⁻¹ h⁻¹) (Bachem, Bubendorf, Switzerland) or isotype-matched control IgG in 0.9% saline for 4 weeks.

Cell Culture

Human pulmonary artery endothelial cells (HPAECs) were purchased from ScienCell Research Laboratories (Carlsbad, Calif) and were cultured in endothelial cell growth medium at 37°C within a humidified atmosphere containing 5% CO₂. For experimental use, the HPAECs were incubated with 2% fetal bovine serum endothelial cell medium for 24 hours, with or without different concentrations of salusin- β peptides (Phoenix Pharmaceuticals, Belmont, Calif).

Hemodynamic Measurements and Evaluation of Right Ventricular Hypertrophy

The Sprague-Dawley rats were anesthetized with 1% pentobarbital sodium (40 mg kg⁻¹ i.p.). As an indicator of mean pulmonary arterial pressure, right ventricular systolic pressure (RVSP) was measured using a guide-wire advanced into the right ventricle via the right jugular vein.¹⁸ The rats were killed by cervical dislocation, the lungs and hearts were harvested, and the intrapulmonary arteries were then isolated. Right ventricular hypertrophy was estimated by calculating the ratio of the right ventricular hypertrophy index (RVHI): RVHI = [right ventricular free wall weight/left ventricular free wall plus interventricular septum weight] $\times$ 100%.

Pulmonary Arterial Morphology Analysis

Specimens of the right lung were fixed with 4% paraformaldehyde (pH 7.4) for 24 hours and then embedded in paraffin wax. Serial sections (5- μ m thickness) were stained with H&E. Percent medial wall thickness (% MT) and medial wall area (% MA), which was used to estimate pulmonary arteriolar remodeling, were calculated as follows: wall thickness (%) = [(medial thickness $\times$ 2)/external diameter] $\times$ 100%; and medial wall area (%) = [outer medial area - luminal area/outer medial area] $\times$ 100%.¹⁹ Images of the pulmonary vessels were captured using a Nikon microscope digital camera system, and the circumferences were measured using its image analysis program (Nikon, Tokyo, Japan).

Immunohistofluorescence and Immunohistochemical Analysis

For double immunofluorescence staining, the lung sections were incubated at 4°C overnight with a mixture of rabbit anti-salusin- β human antibody (1:200 dilution; Phoenix Pharmaceuticals) and anti-CD68 mouse

antibody (1:100 dilution; AbD Serotec, Oxford, UK), anti- α -smooth muscle actin (α -SMA) and anti-von Willebrand factor (vWF) mouse antibody (1:200 dilution; Abcam, Cambridge, UK), or substitution of the primary antibody by an isotype control. Immunohistofluorescence images were obtained via confocal microscopy (TCS-SP5, Leica Microsystems, Wetzlar, Germany). Immunohistochemical analysis was performed as described previously.¹⁸ The sections were then again incubated with rabbit anti-NF- κ B p65 monoclonal antibody (1:200 dilution; Cell Signaling Technology, Danvers, Mass) overnight at 4°C and visualized under a Nikon microscope digital camera system (Nikon).

Western Blot Analysis

Total lung tissues or isolated intrapulmonary arterioles (IPAs) were sonicated in radioimmunoprecipitation assay buffer (Aidlab, Beijing, China) and homogenized. HPAEC nuclear proteins were extracted using a Nuclear Protein Extraction Kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. The protein samples were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Bedford, Mass). The primary antibodies used were as follows: rabbit anti-salusin- β human polyclonal antibody (1:300 dilution; Phoenix Biotechnology, San Antonio, Tex), rabbit anti-NF- κ B p65, anti-phospho-NF- κ B p65, and anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha ($\text{I}\kappa\text{B}\alpha$) and anti-phospho- $\text{I}\kappa\text{B}\alpha$ polyclonal antibody (1:1000 dilution; Cell Signaling Technology). The expression of the proteins was then determined using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) and quantified as relative fold to the expression in the sham-operated group after normalization with GAPDH (1:2000 dilution; Sigma-Aldrich) or Lamin B1 (1:500 dilution; Proteintech, Rosemont, Ill).

Real-Time PCR

The lung tissue or HPAEC total RNA was extracted using the RNeasy Pure Kit (Qiagen Biotech, Beijing, China) according to the manufacturer's instructions. The first-strand cDNA was reverse transcribed from the total RNA using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, Calif). Real-time PCR was performed in an Mx3000P Real-Time PCR Detection System (Agilent, Santa Clara, Calif). The expression of TOR2A, monocyte chemoattractant protein (MCP)-1, CD68, interleukin (IL)-1 β , IL-6, and IL-8 was normalized to that of β -actin as the product of a housekeeping gene. The specificity of the reaction was determined by the detection of the $2^{-\Delta\Delta\text{CT}}$ of the amplification products immediately after the last reaction cycle.²⁰ The forward and reverse primers used are shown in Tables E2 and E3.

Endothelial Cell Adhesion Assay

The confluent HPAECs were incubated with different doses of salusin- β or N-acetylcysteine (NAC; Sigma-Aldrich) for 24 hours. For the pretreatment, the cells were gently trypsinized in PBS and replanted on 96-well microplates (1×10^5 cells/0.2 mL per well) precoated with human fibronectin (20 $\mu\text{g}/\text{mL}$; BD Bioscience, San Jose, Calif), and incubated with different doses of salusin- β for another 30 minutes. If needed, 1 mmol/L NAC was added 30 minutes before the addition of salusin- β . The adhesion of the cells was observed under an inverted microscope (Nikon). The cell adhesion was then measured using ImageJ 1.48.

Endothelial Cell Tube Formation Assay

The 96-well microplates were coated with 30 μL of growth factor-reduced Matrigel (BD Biosciences) and solidified for 30 minutes at 37°C. HPAECs were trypsinized and resuspended at $5 \times 10^5/\text{mL}$, and 0.2 mL of this cell suspension was then added into each well. Tube formation was observed under an inverted microscope (Nikon). Tube length was measured using ImageJ 1.48.

Statistical Analyses

Data are presented as means $\pm$ SD and were analyzed using Prism 5 (GraphPad Software, La Jolla, Calif). Comparisons of parameters between groups were performed using one-way ANOVA, followed by a Newman-Keuls test. The relationship between the time of MCT induction and salusin- β^+ vWF $^+$ or salusin- β^+ CD68 $^+$ expression was analyzed using Spearman rank correlation analysis. The correlation coefficient (R) was calculated to measure the correlation degree between the time of MCT induction and salusin- β^+ vWF $^+$ or salusin- β^+ CD68 $^+$ expression. $P < .05$ was considered as statistically significant.

RESULTS

MCT-Induced Salusin- β Expression in Rat Lung Tissue

Rat models of MCT-induced PAH were established and showed elevated RVSP and RVHI levels at both 2 and 4 weeks ($n = 8$ each group) (Figure E1). In the present study, we used immunohistofluorescence staining, western blot, and real-time PCR to detect salusin- β expression in the lungs of the MCT-treated rats. Immunofluorescence co-localization analysis showed that salusin- β was localized predominantly in the macrophages and vascular endothelium in the rat lung tissue (Figure 1, C and D). The numbers of salusin- β^+ CD68 $^+$ cells per high-power field at 0, 2, and 4 weeks were 3.09 ± 1.56 , 13.53 ± 2.21 , and 24.41 ± 4.32 , respectively ($P < .05$, $n = 6$ each group). There was an obvious linear trend between time of MCT induction and salusin- β^+ CD68 $^+$ expression in the lung tissues ($R^2 = 0.9032$, $P < .05$; Figure 1, A). Moreover, the number of salusin- β^+ vWF $^+$ cells per high-power field in the rats with MCT-induced PAH at 2 and 4 weeks was higher than that at 0 weeks (3.88 ± 1.49 and 5.59 ± 1.37 vs 1.17 ± 1.28 , $P < .05$, $n = 6$ each group). In addition, there was an obvious linear trend between the time of MCT induction and salusin- β^+ vWF $^+$ expression in the lung tissues ($R^2 = 0.6041$, $P < .05$; Figure 1, B). However, salusin- β was not expressed in the pulmonary arterial smooth muscle cells of the rats (Figure E2). We also observed that preprosalusin (salusin- β precursor) expression in the lungs after MCT induction was markedly higher than that before induction (0 weeks) (by approximately 3.17-fold at 2 weeks and 4.12-fold at 4 weeks, respectively, $n = 3$ each group, $P < .05$; Figure 1, E and F). Further, TOR2A transcript levels were significantly increased in the lungs of the MCT-treated rats (by approximately 1.69-fold at 2 weeks and 2.43-fold at 4 weeks, respectively, $n = 3$ each group, $P < .05$; Figure 1, G).

Salusin- β Blockade Ameliorated the Development of PAH and Right Ventricular Hypertrophy in the MCT-Treated Rats

RVSP was significantly higher in the MCT (83.18 ± 7.10 mm Hg, $n = 12$) and MCT + IgG groups (80.59 ± 13.24 mm Hg, $n = 6$) than in the control group (24.56 ± 3.64 mm Hg, $n = 6$, $P < .05$), but it was

FIGURE 1. Salusin- β expression is increased in the lung tissue of MCT-treated rats. A, Salusin- β was expressed on the macrophages in the lungs of the rats with MCT-induced PAH. Box-and-whisker plots show the distributions of salusin- β^+ macrophages per high-power field (20 $\times$ objective). A linear trend between the time of MCT induction and salusin- β^+ CD68 $^+$ expression is shown. B, Salusin- β was expressed on the PAECs in the lungs of rats with MCT-induced PAH. Box-and-whisker plots show the distributions of salusin- β^+ endothelial cells per high-power field (20 $\times$ objective). A linear trend between the time of MCT induction and salusin- β^+ vWF $^+$ expression is shown. C, Representative immunofluorescence images from lung sections stained with salusin- β (green) and CD68 (red) in rats treated with MCT at 0, 2, and 4 weeks. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). D, Representative immunofluorescence images from lung sections stained with salusin- β (green) and vWF (red) in rats treated with MCT at 0, 2, and 4 weeks, and the nuclei stained with DAPI (blue) are shown. E, Preprosalusin (salusin- β precursor) expression was measured using western blotting in the lung tissue lysates. F, Protein level was quantified as its expression relative to that in the control. $*P < .05$. G, TOR2A mRNA levels were measured using real-time PCR in the lung tissues, $*P < .05$. Data are shown as box-and-whisker plots. Sal- β , Salusin- β ; vWF, von Willebrand factor; MCT, monocrotaline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

significantly decreased in the MCT + anti-salusin- β IgG group (40.09 ± 2.37 mm Hg, $n = 6$, $P < .05$; Figure 2, B). Similarly, RVHI was higher in the MCT ($33.2\% \pm 8.32\%$, $n = 12$) and MCT + IgG groups ($28\% \pm 1.87\%$, $n = 6$) than in the control group ($18\% \pm 2.76\%$, $P < .05$), but it was significantly resolved in the MCT + anti-salusin- β IgG group ($20.17\% \pm 2.64\%$, $n = 6$, $P < .05$ vs MCT + IgG group) (Figure 2, C). However, the systemic pressure did not change significantly with chronic anti-salusin- β infusion (Table E1).

Salusin- β Blockade Alleviated MCT-Induced Pulmonary Vascular Remodeling

H&E and immunohistochemistry staining with anti- α -SMA and anti-vWF antibody showed that the pulmonary arterial medium was hypertrophied in the MCT and MCT + IgG groups and that the change was ameliorated in the MCT + anti-salusin- β IgG group (Figure 2, D). The relative medial wall thickening (% MT) and medial wall area (% MA) were significantly higher in the MCT group ($46.73\% \pm 6.03\%$ and $79.14\% \pm 9.11\%$, $n = 12$) and the MCT + IgG group ($50.36\% \pm 7.97\%$ and

FIGURE 2. Salusin-β blockade prevented the development of PAH and pulmonary vascular remodeling. A, Experimental protocol for examining the effect of anti-salusin-β treatment on MCT-induced PAH. MCT-injected rats were treated with anti-salusin-β IgG or isotype-matched control IgG at 4 weeks, and the lungs and hearts were harvested at day 28 for the following analyses; (B) right ventricular systolic pressure (RVSP); (C) RVHI analysis of the PAH rats treated with anti-salusin-β IgG or IgG 4 weeks after MCT challenge. **P* < .05. D, H&E staining (*upper panels*) and immunofluorescence images (*lower panels*) from lung sections stained with vWF (*red*) and α-SMA (*green*) in the control, MCT, MCT + IgG, and MCT + anti-salusin-β IgG groups. The nuclei stained with DAPI (*blue*) are shown. E and F, Pulmonary artery medial wall thickness and medial wall area were measured in the PAH rats treated with anti-salusin-β IgG or IgG 4 weeks after MCT challenge. The external diameter and medial wall thickness of the pulmonary arterioles (ranging in size from 25 to 150 μm in external diameter) were measured using the H&E-stained lung sections. For each artery, the medial wall thickness was expressed as follows: wall thickness (%) = [(medial thickness × 2)/external diameter] × 100%; and medial wall area (%) = [(outer medial area-luminal area)/outer medial area] × 100%. Data are shown as box-and-whisker plots. **P* < .05. MCT + IgG, MCT + isotype-matched control IgG. MCT, Monocrotaline; IgG, immunoglobulin G; RVSP, right ventricular systolic pressure; RVHI, right ventricular hypertrophy index; vWF, von Willebrand factor; α-SMA, α-smooth muscle actin.

FIGURE 3. Salusin- β blockade alleviated macrophage infiltration and mRNA expression of inflammatory cytokines in the lungs of rats with MCT-induced PAH. A, Representative immunohistochemical images of pulmonary macrophages stained for CD68 in the lungs of rats treated with MCT are shown. B, Quantitative analysis of macrophage expression. Data are shown as box-and-whisker plots. Expression of CD68 (C), MCP-1 (D), IL-1 β (E), and VCAM-1 (F) in the lung tissues, as measured using real-time PCR. Values are normalized to β -actin expression levels. Data are shown as box-and-whisker plots. * $P < .05$. MCT, Monocrotaline; IgG, immunoglobulin G; IL-1 β , interleukin-1 β ; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

81.79% $\pm$ 5.73%, $n = 6$) than in the control group (15.69% $\pm$ 3.83% and 43.74% $\pm$ 8.60%, $n = 6$, $P < .05$), but significantly reduced in the MCT + anti-salusin- β IgG group (30.19% $\pm$ 5.62% and 49.57% $\pm$ 8.29%, $n = 6$, $P < .05$ vs the MCT + IgG group; Figure 2, E and F).

Salusin- β Blockade Attenuated Macrophage Infiltration and Inflammatory Cytokine Expression in the Lungs of Rats With MCT-Induced PAH

Macrophage infiltration and secreted pro-inflammatory cytokine expression were closely associated with the development of MCT-induced PAH.²¹ There was more macrophage infiltration in the MCT and MCT + IgG groups than in the control group, and this effect was suppressed by anti-salusin- β IgG treatment ($n = 6$ each group, $P < .05$), as indicated by immunohistochemical analyses (Figure 3, A and B). Similarly, the mRNA expression of CD68, IL-1 β , MCP-1, and vascular cell adhesion molecular-1 (VCAM-1) was higher in the MCT and MCT + IgG groups than in the control group, but the expression was reduced by salusin- β blockade ($P < .05$, $n = 3$ each group; Figure 3, C-F).

Salusin- β Blockade Inhibited NF- κ B Activation in the Lungs and Pulmonary Arterioles of Rats With MCT-Induced PAH

Immunohistochemical analysis showed that NF- κ B expression was significantly increased in the pulmonary arterioles of the MCT-treated rats and those treated with MCT + IgG, but decreased to a significant degree in the rats treated with MCT + anti-salusin- β ($n = 6$ each group) (Figure 4, A). Similarly, western blot analysis showed phosphorylation and degradation of I κ B α and activated phosphorylation of NF- κ B p65 and upregulation of NF- κ B p65 protein expression in the lungs and pulmonary arteries of the rats treated with MCT and MCT + IgG, but decreased activated phosphorylation of NF- κ B p65 and upregulation of NF- κ B p65 protein expression in the rats treated with MCT + anti-salusin- β ($n = 3$ each group, $P < .05$; Figure 4, B-F).

Salusin- β Stimulated HPAEC Adhesion

One of the major clinical and pathologic features of PAH is dysfunction of the PAECs.²² We therefore performed adhesion experiments in vitro to further assess the effects of salusin- β on endothelial cell dysfunction. Cultured

FIGURE 4. Salusin- β blockade inhibited NF- κ B activation in the lungs and pulmonary arterioles of rats with MCT-induced PAH. A, Localization of NF- κ B was assessed using immunohistochemistry in the pulmonary arterioles of the rats with MCT-induced PAH treated with anti-salusin- β IgG or IgG 4 weeks after MCT challenge. B, Western blot analysis of the protein expression of phospho-I κ B α , I κ B α , phospho-NF- κ B p65, NF- κ B p65, and GAPDH in the lung tissues and IPAs. Statistical analysis of the expression of (C) phospho-I κ B α /I κ B α , (D) phospho-NF- κ B/NF- κ B in the lung tissues; (E) phospho-I κ B α /I κ B α and (F) phospho-NF- κ B/NF- κ B in IPAs; * P < .05. MCT, Monocrotaline; IgG, immunoglobulin G; I κ B α , nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor, alpha; NF- κ B, nuclear factor kappa light chain enhancer of activated B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPAs, intrapulmonary arterioles.

FIGURE 5. Salusin- β stimulated HPAEC adhesion. A, Effects of salusin- β on the adhesion of cultured HPAECs. Cells were pretreated with the 1 mM NAC for 10 minutes and then stimulated with 20 nM Sal- β for 30 minutes. Scale bar represents 100 μ m. B, Quantitation of the data in (A). $n = 6$ each group, $*P < .05$. Data are shown as box-and-whisker plots. $*P < .05$; $n = 3$ each group. Sal- β , Salusin- β ; NAC, N-acetylcysteine.

HPAECs were stimulated with salusin- β at different concentrations (1, 10, and 20 nmol/L, $n = 6$ each group) for 24 hours. Treatment of the HPAECs with salusin- β significantly increased the number of adherent cells in vitro. However, pretreatment with the NF- κ B inhibitor, NAC,²³ suppressed salusin- β -induced cell adhesion ($P < .05$, $n = 6$; Figure 5, A and B).

Salusin- β Accelerated HPAEC Tube Formation and Increased the Expression of Pro-Inflammatory Cytokines by Activation of NF- κ B Signaling

The tube formation assay showed that salusin- β (10 and 20 nmol/L) induced an increase in tube formation (1.5-fold and 2-fold) in the HPAECs cultured in Matrigel compared with that in the control group ($n = 6$ each group; Figure 6, A and B). Moreover, NF- κ B inhibition attenuated the salusin- β -induced tube formation. Western blot analysis showed that the salusin- β -induced nuclear translocation of the NF- κ B subunit p65 was significantly reversed by NAC ($n = 3$ each group, $P < .05$; Figure 6, C and D). Real-time PCR results showed that salusin- β treatment significantly induced the expression of IL-1 β and IL-6, and that this effect was abolished by NAC. However, IL-8 expression was not affected ($n = 3$ each group, $P < .05$; Figure 7, A-C).

DISCUSSION

This study shows that salusin- β is broadly expressed in the pulmonary macrophages and pulmonary arterial endothelial cells (PAECs) of the lungs of MCT-treated rats and that the blockage of endogenous salusin- β by anti-salusin- β IgG suppressed pulmonary inflammation and ameliorated pulmonary vascular remodeling and right ventricular hypertrophy during the development of MCT-induced PAH in the rats. In addition, salusin- β can induce inflammatory

cytokine expression by regulating the I κ B α /NF- κ B pathway to influence PAEC dysfunction associated with PAH (Figure E3).

MCT is a plant pyrrolizidine alkaloid, and the MCT-induced PAH model is a classic model with characteristics of human idiopathic PAH.²⁴ Our previous results indicated that MCT treatment (60 mg/kg) could increase RVSP and result in the pathologic features characteristic of PAH and low mortality at 4 weeks in rats, which is in agreement with our results from the present study.²⁵ The data from the present study also showed that salusin- β expression was increased in the lungs of the MCT-treated rats at 2 and 4 weeks, and that salusin- β was localized in the pulmonary macrophages and PAECs (Figure 1). Moreover, salusin- β blockade could significantly inhibit MCT-induced pulmonary vascular remodeling and alleviate RVSP and right ventricular hypertrophy (Figure 2). Previous studies have suggested that salusin- β can contribute to vascular inflammation and lead to vascular remodeling associated with atherosclerosis and hypertension.^{16,26} Salusin- β has also been shown to be secreted by human monocytes/macrophages in vitro.²⁷ Taken together, our results suggest that salusin- β also contributes to pulmonary vascular remodeling and right ventricular hypertrophy in MCT-treated rats.

Inflammation plays an important role in the pathogenesis of PAH.²⁸ The expression of inflammatory cytokines such as MCP-1, IL-1 β , and IL-6 is increased in several types of PAH, and the levels of cytokines in circulation correlate with disease severity and outcome.²⁸ Macrophages are the main inflammatory infiltrators of plexiform lesions in PAH.²⁹ In the current study, salusin- β inhibition suppressed macrophage infiltration and decreased the mRNA expression of MCP-1, IL-1 β , and VCAM-1 in the lungs

FIGURE 6. Salusin- β accelerated HPAEC tube formation by activation of the NF- κ B pathway. A, Representative images showed the effects of different concentrations of salusin- β on tube formation in the HPAECs for 24 hours. Cells were pretreated with the 1 mM NAC for 30 minutes and then stimulated with 20 nM Sal- β for 24 hours. Scale bar represents 100 μ m. B, Quantitation of the data in (A). $n = 6$ each group, $*P < .05$. C, Representative western blot showing NF- κ B p65 in the nuclear extracts from cultured HPAECs treated with different concentrations of salusin- β for 6 hours. Cells were pretreated with 1 mM NAC for 30 minutes and then stimulated with 20 nM Sal- β for 6 hours using lamin B1 as the loading control. Statistical analysis of the expression of NF- κ B p65/lamin B1 (D). Data are shown as box-and-whisker plots. $n = 3$ each group, $*P < .05$. Sal- β , Salusin- β ; NAC, N-acetylcysteine; NF- κ B, nuclear factor kappa light chain enhancer of activated B.

of the rats with MCT-induced PAH (Figure 3). Moreover, in vitro experiments showed that salusin- β selectively promoted the expression of IL-1 β and IL-6, but not that of IL-8 in the HPAECs (Figure 7). Previously, salusin- β has also been shown to increase MCP-1, TNF- α , IL-1 β , and IL-6 expression in the paraventricular nucleus of rats with spontaneous hypertension.²⁶ The present study suggested that salusin- β contributes to the pulmonary tissue inflammation observed in the MCT-treated rats.

NF- κ B is known to regulate the expression of targeted genes associated with inflammation, the dysfunction of endothelial cells, and angiogenesis.³⁰ Inflammatory stimuli can trigger the degradation of I κ B proteins, causing the release of NF- κ B p65, followed by its translocation to the nucleus, where it modulates target gene transcription and

causes I κ B phosphorylation. Nuclear translocation of NF- κ B p65 is central to processes such as NF- κ B activation.³¹ In the current study, we observed that salusin- β blockade inhibited MCT-induced I κ B phosphorylation and degradation and activation of NF- κ B p65 in the lung tissue and IPAs (Figure 4), and that it induced nuclear translocation of NF- κ B p65 in cultured HPAECs (Figure 6, C and D). Salusin- β can also directly induce vascular inflammation via the I κ B α /NF- κ B pathway in apoE^{-/-} mice.³² In addition, salusin- β treatment has been shown to lead to I κ B α phosphorylation and degradation and nuclear translocation of NF- κ B in cultured human umbilical vein endothelial cells.³³ These results suggest that salusin- β promotes NF- κ B-mediated inflammation in pulmonary vascular and cultured HPAECs. However, recently, some

FIGURE 7. Salusin- β upregulated the expression of inflammatory cytokines in cultured HPAECs. mRNA expression of IL-1 β , (A) IL-6, (B) and IL-8, (C) in cultured HPAECs stimulated with Sal- β for 6 hours. Cells were pretreated with the 1 mM NAC for 30 minutes and then stimulated with 20 nM Sal- β for 6 hours. Data are shown as box-and-whisker plots. $n = 3$ each group, $*P < .05$. Sal- β , Salusin- β ; IL-1 β , interleukin-1 β .

reports have shown that the STAT3/NFAT and TGF- β /Smad signaling pathways can also mediate inflammatory reactions in PAH²¹; however, the effect of salusin- β on these pathways is still not known. Future work will be needed to identify whether salusin- β contributes to vascular inflammation associated with PAH through other inflammatory pathways.

VIDEO 1. The video mainly shows mini-osmotic pumps subcutaneously implanted in the MCT-treated rats in vivo. The video contains 2 parts. The first part shows the subcutaneous implantation of the pumps. Briefly, the pumps were prepared, and empty ALZET pumps were filled with about 200 μ L of anti-salusin- β IgG or isotype-matched control IgG using a syringe. Surgical procedure: the rats were anesthetized using 1% sodium pentobarbital and the area for pump placement was shaved and disinfected with 70% ethanol. A filled pump was then inserted into the shaved area. The skin incision was then closed using wound clips. The second part shows RVSP measurements. RVSP was measured using a guide-wire advanced into the right ventricle via the right jugular vein. Video available at: [http://www.jtcvsonline.org/article/S0022-5223\(16\)30494-9/addons](http://www.jtcvsonline.org/article/S0022-5223(16)30494-9/addons).

One of the key clinical and pathologic features of PAH is the dysfunction of PAECs, which includes enhanced adhesion capacity and uncontrolled angiogenesis, resulting in increased susceptibility of the pulmonary distal arteries to vascular remodeling.³⁴ The data presented here indicate that exogenous salusin- β could enhance cell adhesion capacity and angiogenesis in the cultured HPAECs (Figures 5 and 6). More importantly, NF- κ B inhibition significantly attenuated the salusin- β -induced cell adhesion and angiogenesis in the cultured HPAECs. In addition, salusin- β was broadly expressed in vascular endothelial cells, with the ability to accelerate monocyte/macrophage adhesion to vascular endothelial cells and cause inflammatory responses in them.³³ These findings suggest that salusin- β can induce HPAEC dysfunction associated with inflammation by activating the NF- κ B pathway.

Limitations

As no salusin- β transgenic or knockout animals are available, no specific inhibitor or antagonist of salusin- β has been identified until now. Our study mainly focuses on using the inhibition of endogenous salusin- β to study the role of salusin- β in the pathogenesis of MCT-induced PAH. We found that salusin- β induces inflammatory reactions by activating the I κ B α /NF- κ B pathway and inducing the expression of inflammatory cytokines.

CONCLUSIONS

The current results show that salusin- β contributes to the development of PAH. Salusin- β facilitates HPAEC adhesion and angiogenesis in vitro. Inhibition of salusin- β in the lungs decreases the expression of inflammatory

cytokines through the NF- κ B pathway in the rat model of MCT-induced PAH, thereby alleviating PAH. Targeting salusin- β in the lungs may be a suitable therapeutic method for PAH.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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