

Mast cells promote lung vascular remodeling in pulmonary hypertension

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Word count Body of the manuscript: 3,329 words; Abstract: 198 words

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Abstract

Left heart disease (LHD) frequently causes lung vascular remodeling and pulmonary hypertension (PH). Yet, pharmacological treatment for PH in LHD is lacking, and its pathophysiological basis remains obscure. We aimed to identify candidate mechanisms of PH in LHD, and to test their relevance and therapeutic potential.

In rats, LHD was induced by supracoronary aortic banding. Whole genome microarray analyses were performed, candidate genes were confirmed by RT-PCR and Western, and functional relevance was tested *in vivo* by genetic and pharmacological strategies.

In lungs of LHD rats, "mast cell activation" was the most prominently upregulated gene ontology cluster. Mast cell gene upregulation was confirmed at RNA and protein levels, and remodeled vessels showed perivascular mast cell accumulations. In LHD rats treated with the mast cell stabilizer ketotifen, or in mast cell deficient *Ws/Ws* rats, PH and vascular remodeling were largely attenuated. Both strategies also reduced PH and vascular remodeling in monocrotaline-induced pulmonary arterial hypertension, suggesting that the role of mast cells extends to non-cardiogenic PH.

In PH of different etiologies, mast cells accumulate around pulmonary blood vessels and contribute to vascular remodeling and PH. Mast cells and mast cell-derived mediators may present promising targets for the treatment of PH.

Keywords

Heart failure, mast cells, pulmonary hypertension, vascular remodeling

Introduction

Two thirds of patients with chronic severe left-sided heart disease develop a pulmonary artery pressure (PAP) of 25 mmHg or more at rest [1]. According to the 2009 WHO clinical classification, these patients fall into the second and presumably largest category of pulmonary hypertension (PH), i.e. PH owing to left heart disease (LHD) [2]. In chronic LHD, PH is not only caused by a congestive increase in lung vascular pressures, but is frequently aggravated by a concomitant rise in pulmonary vascular resistance (PVR) [3] which further increases right ventricular afterload, limits right ventricular output, and ultimately promotes right ventricular failure. The clinical relevance of this scenario is underlined by the fact that PH, reduced right ventricular ejection fraction or increased PVR each constitute independent predictors of mortality in chronic LHD [4]. Yet, in contrast to other forms of PH, efficient and/or approved treatment strategies for PH owing to LHD are still lacking.

The pathology of PH owing to LHD is characterized by endothelial dysfunction, increased vessel tone and vascular remodeling as a result of chronically elevated pressures in the pulmonary circulation [5]. In a model of supracoronary aortic banding in rats that replicates these characteristics [6] we aimed to elucidate underlying cellular and molecular mechanisms as a rational basis for the design of new treatment strategies. By genome wide expression analyses we identified potential target genes involved in vascular regulation, inflammatory or developmental pathways. Notably, the strongest differential regulation was an upregulation of genes associated with mast cell activation. Based on this finding we hypothesized a putative role for mast cells in vascular remodeling and PH owing to LHD, and potentially in other, non-cardiogenic forms of PH as well. We tested this hypothesis both by pharmacological intervention and in a genetic model of mast-cell deficient rats. Our findings identify mast cells as critical promoters of lung vascular remodeling in PH of different etiologies, and suggest mast cell-targeted strategies as promising interventions for the treatment of PH.

Methods

A detailed material and method section is provided as online supplementary material. In brief, PH owing to left heart disease was induced by supracoronary aortic banding in Sprague-Dawley rats, mast cell-deficient *Ws/Ws* rats or corresponding BN/fMai wild types as described [6]. Sham operated rats served as controls. Non-cardiogenic PH was induced by intraperitoneal injection of monocrotaline (MCT; 60 mg/kg in Sprague-Dawley and 40 mg/kg in *Ws/Ws* or BN/fMai rats, respectively). Ketotifen (1 mg/kg bw/day) was given with the drinking water. Cardiopulmonary characterization was performed nine weeks after aortic banding or sham operation, and three weeks after MCT injection as described [6]. Using echocardiographic transthoracic 2-dimensional (2D) and M-mode imaging, right and left ventricular end-diastolic areas were determined, and tricuspid annular plane systolic excursion (TAPSE) was measured for evaluation of right ventricular systolic longitudinal function.

Whole rat genome microarray analyses of lungs from banded and control rats and subsequent bioinformatic analyses were performed as outlined in the supplementary material. Differentially expressed genes were visualized by heat-maps, and analysed for enrichments of Gene Ontology (GO) classes. For selected genes, differential expression was confirmed at the transcriptional and translational level by real-time RT-PCR and Western blot analysis. Vascular remodelling, mast cell accumulation and degranulation were quantified by blinded analysis of histological sections as described in the supplementary material. In brief, medial wall thickness was quantified in small (20-50 μm diameter), medium sized (50-100 μm) and larger (>100 μm) pulmonary arterioles, and the fraction of non-muscularized, partially muscularized or fully muscularized vessels as well as the vascular lumen area were determined in small arterioles. Density of mast cells was measured in toluidine blue-stained lung sections, and the number of perivascular mast cells surrounding each vessel was determined for different vessel calibers. An index of granulation (IOG) was calculated as ratio of granulated over degranulated mast cells. All data are given as means \pm SEMs, and statistical significance was assumed at $p < 0.05$.

Results

Whole rat genome microarray analyses were performed in lung homogenates of three rats with established PH following supracoronary aortic banding and in three sham-operated controls. The complete microarray data set is accessible at the Gene Expression Omnibus (GEO) repository of the National Center for Biotechnology Information with the accession no. GSE16624 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16624>). Of a total of 28,000 analyzed genes, differential expression defined as ≥ 4 fold change with $p < 0.05$ was evident for 120 genes. Of these, 76 were up- and 44 downregulated in aortic banding as compared to control lungs as illustrated by volcano blot (Fig. 1A). Differentially regulated genes given as heat map in Fig. 1B encode proteins involved in inflammatory and developmental pathways, complement and coagulation cascades, vascular regulation, or extracellular matrix composition (Table 1). Yet, the gene ontology (GO) class with the most pronounced differential regulation was the GO class "mast cell activation" in which 13 out of 20 genes were significantly upregulated in aortic banding as compared to control lungs (Fig. 1C). The notion of an increased expression of mast cell genes in PH owing to left heart disease was confirmed for selected candidate genes. RT-PCR (Fig. 1D) and Western blot analyses (Fig. 1E) demonstrate upregulation of mast cell chymase and mast cell peptidase 2 in aortic banding lungs at both the genomic and proteomic level.

In order to test for a putative functional role of mast cells in lung vascular remodeling and PH in LHD, we applied both a pharmacological approach by treatment of aortic banding rats with the mast cell stabilizer ketotifen and a genetic strategy by use of *Ws/Ws* rats which are deficient in mast cells [7]. Ketotifen treatment from postoperative day 1 significantly attenuated the development of PH and right ventricular hypertrophy over 9 weeks of aortic banding, as demonstrated by a reduced PAP and right ventricular weight in ketotifen-treated as compared to untreated banded rats (Fig. 2A). In sham-operated control rats without banding, ketotifen treatment had no detectable effect on pulmonary hemodynamics. The attenuation of PH by ketotifen was predominantly attributable to a normalization of PVR, while left atrial pressure and aortic flow did not differ significantly between ketotifen treated and untreated rats (supplementary table 2), indicating that ketotifen did not mitigate underlying congestive heart failure. Consistent with our previous findings [6], arterial pressure and systemic vascular resistance did not differ between banded and control rats and were similarly unaffected by ketotifen treatment (supplementary table 2). Echocardiographic analyses 9 weeks after aortic

banding revealed an increased right ventricular end-diastolic area as compared to controls consistent with the development of cor pulmonale (Figs. 2B,C). Ketotifen treatment largely normalized right ventricular end-diastolic dimensions to control values. Left ventricular end-diastolic area showed a trend to increase in rats with aortic banding independent of ketotifen treatment, yet without reaching significance. TAPSE was reduced in aortic banding rats as compared to controls, indicating right ventricular dysfunction, but was restored in part by ketotifen treatment (Fig. 2C).

Considerable lung vascular remodeling was evident in banded rats as medial wall thickening in all analyzed vessel categories, i.e. in small and medium-sized pulmonary arterioles of 20-50 μm and 50-100 μm diameter, respectively, as well as in larger arterioles of >100 μm diameter (Figs. 3A,B). While the majority of small pulmonary arterioles was non-muscularized or partially muscularized in control rats, a marked increase in fully muscularized arterioles was evident in banded rats and was associated with a concomitant decrease in vascular lumen area (Fig. 3C). Ketotifen treatment significantly attenuated the increase in medial wall thickness in all analyzed vessel categories, reduced microvascular muscularization, increased vascular lumen area and thus, largely prevented lung microvascular remodeling in banded rats (Figs. 3A-C). Significant mast cell accumulation and degranulation in lungs of banded as compared to control rats was evident as increased mast cell density, reduced granularity, and perivascular mast cell accumulation in all analyzed vessel calibers (Figs. 3D). Mast cells did not colocalize with the occasional occurrence of vascular thrombosis or microvascular hemorrhage in the banding model, suggesting that these pathologies did not act as the primary trigger for mast cell recruitment. In line with its proposed mode of action as a mast cell stabilizer, ketotifen treatment prevented mast cell degranulation and increased granularity even above control levels. In addition, ketotifen attenuated the increase in lung mast cell density, and prevented in particular the perivascular accumulation of mast cells around small pulmonary microvessels of 20-50 μm diameter (Fig 3D).

In a second, non-pharmacological approach we addressed the role of mast cells by subjecting mast cell deficient *Ws/Ws* and corresponding *BN/fMai* wild type rats to 9 weeks of aortic banding. As compared to wild types, the increase in PAP and PVR following aortic banding was significantly attenuated in *Ws/Ws* rats (Fig. 4A). Likewise, right ventricular hypertrophy was reduced (Fig. 4A), and vascular remodeling, i.e. the increase in medial wall thickness and

muscularization and the loss of vessel lumen, was markedly attenuated in banded *Ws/Ws* as compared to wild type rats (Figs. 4B,C). Sham operated *Ws/Ws* and wild type rats did not differ in any of the assessed parameters. In line with the results obtained in Sprague-Dawley rats, aortic banding resulted in pulmonary mast cell accumulation and degranulation in wild type BN/fMai rats. In agreement with their reported phenotype, no mast cells were detectable in lung sections from *Ws/Ws* rats (Fig. 4D).

To test whether the role of mast cells also expands to non-cardiogenic forms of PH, we analyzed the effects of ketotifen in MCT-induced pulmonary arterial hypertension. Within 3 weeks after MCT injection, Sprague-Dawley rats developed PH and lung vascular remodeling as demonstrated by increased PAP and PVR, right ventricular hypertrophy (Fig. 5A), and an increase in mean pulmonary arteriolar wall thickness (Fig. 5B), that was associated with an accumulation and degranulation of mast cells (Fig. 5C). Analogous to our findings in banded rats, ketotifen treatment mitigated these effects, and attenuated the MCT-induced increase in right ventricular end-diastolic area as assessed by echocardiography (Fig. 5B). Left ventricular end-diastolic area was not altered by either MCT or ketotifen treatment. To substantiate the notion that mast cells may be equally relevant in pulmonary arterial hypertension, we next compared the effects of MCT in mast cell deficient *Ws/Ws* and wild type BN/fMai rats. As BN/fMai rats showed the characteristic features of the MCT model, i.e. an increased PAP and PVR, right ventricular hypertrophy, echocardiographic signs of cor pulmonale and right ventricular dysfunction, and extensive vascular remodeling, these effects were markedly attenuated or even abrogated in mast cell-deficient *Ws/Ws* rats (Figs. 6A,B). These findings consolidate the view that the role of mast cells in lung vascular remodeling and PH is not restricted to left heart disease, but extends to other categories of PH, and may as such constitute a unifying disease mechanism independent of the individual etiology.

Discussion

Here, we identify mast cells as catalysts of lung vascular remodeling in PH. Gene array analyses identified "mast cell activation" as most prominently upregulated GO class in PH subsequent to aortic banding, and perivascular mast cell accumulation was substantiated on the genomic, proteomic and histological level. A functional role of mast cells in PH owing to LHD was demonstrated by a dual pharmacological and genetic strategy in that both, the mast cell stabilizer ketotifen or the use of mast cell deficient *Ws/Ws* rats attenuated (i) PH, (ii) the increase in PVR, (iii) lung vascular remodeling, (iv) right ventricular hypertrophy and (v) end-diastolic right ventricular dilation in aortic banding rats. The protective effects of ketotifen or mast cell deficiency, respectively, were likewise evident in a model of MCT-induced pulmonary arterial hypertension, suggesting that the role of mast cells expands to other forms of PH. Mast cells may thus present a novel and promising target in the treatment of PH.

Whereas mast cells have traditionally been recognized as critical in allergic and non-allergic immune responses, a growing body of evidence implicates these cells in cardiovascular disease. Mast cells stimulate the proliferation of endothelial [8] and smooth muscle cells [9] which present hallmarks of PH. In the present study, the upregulation of mast cell genes and the accumulation of mast cells in heart failure lungs let us to speculate on a pathophysiological role for mast cells in vascular remodeling and PH. Notably, the notion that mast cells accumulate in lungs in LHD is not new. Paul Ehrlich, who first identified this cell type in 1878, noted that mast cells were abundant in "brown induration of the lung", i.e. in hemosiderosis following mitral stenosis [10]. Since then, several studies have confirmed the accumulation of mast cells in lungs of patients with PH secondary to congenital cardiac septal defects or mitral stenosis as well as in patients with idiopathic PH [11,12]. These clinical findings were paralleled by similar observations in experimental models of PH [13,14]. Although these studies cohesively proposed that the accumulation of mast cells in the lung may have potential functional relevance for the pathophysiology of PH and lung vascular remodeling, this hypothesis has never been systematically tested as yet.

Several lines of argument led us to propose that mast cells may play a critical role in PH: Mast cells release biogenic amines including serotonin which plays a key role in pulmonary arterial vasoconstriction and smooth muscle cell proliferation [15] or histamine which is a

vasoconstrictor in pulmonary veins. Mast cells activate the renin-angiotensin system which has been implicated in the pathogenesis of PH [16] in that they release renin and mast cell chymase which converts angiotensin I to angiotensin II [17]. Mast cell chymase also processes pro-matrix metalloprotease 9 (pro-MMP-9) to active MMP-9 which is a biomarker for scleroderma-associated PH [18]. Mast cells furthermore produce collagen-cleaving MMP-13 [19] as well as platelet-derived growth factor and transforming growth factor- β (TGF- β) which both stimulate smooth muscle cell proliferation [20]. Mast cells secrete activin A [9], a member of the TGF- β superfamily that is elevated in PH and again promotes smooth muscle cell proliferation. Last not least, mast cells release interleukin-6 (IL-6) which plays a critical role in animal models of PH [21] and is elevated in serum of patients with idiopathic PH [22]. Taken together, these data not only consolidate the hypothesis that mast cells may contribute to PH, but provide a potential mechanistic basis for this notion.

Remarkably, the postulated role for mast cells may also be at the bottom of or at least contribute to the effectiveness of kinase inhibitors in the treatment of PH. Mast cells express high levels of the tyrosine kinase stem cell factor receptor c-kit which regulates their maturation, differentiation and activation [23]. Importantly, multikinase and tyrosine kinase inhibitors such as sorafenib and imatinib which have recently emerged as powerful new treatment strategies for PH [24,25] are potent inhibitors of c-kit [26], and as such, may exert their protective effects at least in part by inhibition of mast cell maturation and function.

Based on these concurrent data, we postulated a functional role for mast cells in lung vascular remodeling and PH. In the first set of experiments we tested this hypothesis by treating rats after aortic banding with the mast cell stabilizer ketotifen. Notably, ketotifen is clinically approved as oral therapy for asthma in children. Ketotifen treatment effectively attenuated the development of PH, lung vascular remodeling, and right ventricular hypertrophy over 9 weeks after banding. Notably, ketotifen not only blocked mast cell degranulation, but equally mitigated mast cell accumulation in the lung parenchyma and around pulmonary blood vessels, suggesting that mast cell activation and accumulation may mutually promote each other in PH. Yet, some caution as to the specificity of this pharmacological intervention is warranted, since ketotifen is not only a mast cell stabilizer, but also a histamine H1-receptor antagonist, and has been proposed to have additional off-target effects which may include reduced phosphodiesterase and increased

methyltransferase activity as well as calcium antagonistic properties [27,28]. Therefore, we applied a second, genetic strategy to substantiate a potential role of mast cells in PH.

Ws/Ws rats possess two mutant alleles at the white spotting (*Ws*) locus which is identical with the *c-kit* proto-oncogene. As a consequence, suckling *Ws/Ws* rats are deficient in mast cells and severely anemic due to a defect of mast cell and erythrocyte precursors. The magnitude of anemia is abated in adult *Ws/Ws* rats, while that of mast cell deficiency is enhanced, making this mutant a useful model for the study of mast cell functions [7]. In the present study, hematocrit did not differ between *Ws/Ws* and corresponding BN/fMai wild type rats at the time of investigations (supplementary table 3), while *c-kit* positive cells were completely absent in histological lung sections from *Ws/Ws* rats (supplementary Fig. 1). In banded rats, the effects of the *c-kit* mutation paralleled those of ketotifen, in that PH, lung vascular and right ventricular remodeling were mitigated in *Ws/Ws* rats. The notion that *c-kit* positive mast cells accumulate in the lung in PH is in line with previous findings in a bovine model of hypoxia-induced PH demonstrating the mobilization of *c-kit*⁺ cells from the bone marrow into the circulation, and their accumulation in the remodeled pulmonary arterial vessel walls [29]. The fact that the receptor tyrosine kinase *c-kit* is essential for the maturation, differentiation and activation of mast cells may furthermore provide an alternative mechanistic explanation for the emerging therapeutic potency of multikinase and tyrosine kinase inhibitors in the treatment of PH [24,25]. Yet, expression and function of *c-kit* are not exclusive to mast cells, but apply to hematopoietic precursor cells and stem cells, germ cells, melanocytes, Cajal cells, and a considerable number of neoplasms [30]. While the attenuation of vascular remodeling and PH in *Ws/Ws* rats alone can thus not be unequivocally attributed to their mast cell deficiency, we believe that the concurrent results obtained by both a pharmacological mast cell stabilizer and in the genetic model of a *c-kit* mutant rat provide substantial evidence for a functional role of mast cells in lung vascular remodeling.

Since pulmonary accumulation of mast cells is a hallmark not only of left heart disease, but similarly of other forms of PH, we used the monocrotaline model to test whether this concept also applies to non-cardiogenic PH. Analogous to their effects in banded rats, ketotifen treatment or genetic mast cell deficiency attenuated MCT-induced pulmonary vascular, hemodynamic and right ventricular changes suggesting that mast cells may also play a critical role in pulmonary arterial hypertension. Notably, prevention of mast cell degranulation by disodium cromoglycate was recently shown to attenuate the development of hypoxic PH in rats [31], while mast cell

deficiency did not prevent PH and vascular remodeling in W/W^u mice which similar to Ws/Ws rats have two mutant alleles at the *c-kit* locus [32]. While this apparent difference between species may be attributable to the relative paucity of mast cells in the mouse [32], it raises the question to which degree data from animal models reflect the clinical situation in humans. The abundance of mast cells in lungs of patients with PH [11,12], and the fact that humoral mediators secreted from human mast cells stimulate proliferation of human smooth muscle cells [20] support the notion that mast cells also play a fundamental role in PH in humans. Yet, this view will ultimately have to be put to the test in appropriate clinical trials.

Our mechanistic insights into the emerging role of mast cells in non-allergic diseases are still scarce. Future studies both *in vivo* and *in vitro* are essential to advance our understanding of the how, when and where mast cells contribute to vascular remodeling and PH. In view of the abundance of potential candidate pathways outlined above, a detailed dissection of intra- and intercellular signaling events pertinent to the role of mast cells in PH was beyond the scope of this study. Importantly, the present recognition of the mast cell as a critical promoter of lung vascular remodeling may give rise to a new spectrum of therapeutic strategies ranging from clinically approved mast cell stabilizers to inhibitors of specific constituents of the mast cell releasate or interventions targeted to prevent mobilization of bone marrow-derived mast cells or mast cell precursors or to block mast cell adhesion and recruitment to the lung.

Acknowledgements

We wish to thank Ewa Bieniek, Argid and Raimund Rutenberg for excellent technical assistance. This study received financial support from the European Commission under the 6th Framework Programme (Contract No: LSHM-CT-2005-018725 Pulmotension to IS, RS, WMK), the Heart and Stroke Foundation of Ontario (NA 6900 to WMK), and fellowships of the Kaiserin Friedrich Foundation Berlin (JY, NY).

References

1. Ghio S, Gavazzi A, Campana C, Inserra C, Klersy C, Sebastiani R, Arbustini E, Recusani F, Tavazzi L. Independent and additive prognostic value of right ventricular systolic function and pulmonary artery pressure in patients with chronic heart failure. *J Am Coll Cardiol* 2001; 37: 183-188.
2. Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, Elliott CG, Gaine SP, Gladwin MT, Jing ZC, Krowka MJ, Langleben D, Nakanishi N, Souza R. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 2009; 54: S43-S54.
3. Lam CS, Roger VL, Rodeheffer RJ, Borlaug BA, Enders FT, Redfield MM. Pulmonary hypertension in heart failure with preserved ejection fraction: a community-based study. *J Am Coll Cardiol* 2009; 53: 1119-1126.
4. Delgado JF, Conde E, Sanchez V, Lopez-Rios F, Gomez-Sanchez MA, Escribano P, Sotelo T, Gomez de la Camara A, Cortina J, de la Calzada CS. Pulmonary vascular remodeling in pulmonary hypertension due to chronic heart failure. *Eur J Heart Fail* 2005; 7: 1011-1016.
5. Kerem A, Yin J, Kaestle SM, Hoffmann J, Schoene AM, Singh B, Kuppe H, Borst MM, Kuebler WM. Lung endothelial dysfunction in congestive heart failure. Role of impaired Ca²⁺ signaling and cytoskeletal reorganization. *Circ Res* 2010; 106: 1103-1116.
6. Yin N, Kaestle S, Yin J, Hentschel T, Pries AR, Kuppe H, Kuebler WM. Inhaled nitric oxide versus aerosolized iloprost for the treatment of pulmonary hypertension with left heart disease. *Crit Care Med* 2009; 37: 980-986.
7. Niwa Y, Kasugai T, Ohno K, Morimoto M, Yamazaki M, Dohmae K, Nishimune Y, Kondo K, Kitamura Y. Anemia and mast cell depletion in mutant rats that are homozygous at "white spotting (Ws)" locus. *Blood* 1991; 78: 1936-1941.
8. Marks RM, Roche WR, Czerniecki M, Penny R, Nelson DS. Mast cell granules cause proliferation of human microvascular endothelial cells. *Lab Invest* 1986; 55: 289-294.
9. Cho SH, Yao Z, Wang SW, Alban RF, Barbers RG, French SW, Oh CK. Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells. *J Immunol* 2003; 170: 4045-4052.

10. Ehrlich P. Beiträge zur Kenntnis der granulierten Bindegewebszellen und der eosinophilen Leukozyten. *Arch Anat Physiol* 1879; 3: 166-169.
11. Heath D, Trueman T, Sukonthamarn P. Pulmonary mast cells in mitral stenosis. *Cardiovasc Res* 1969; 3: 467-471.
12. Heath D, Yacoub M. Lung mast cells in plexogenic pulmonary arteriopathy. *J Clin Pathol* 1991; 44: 1003-1006.
13. Kay JM, Waymire JC, Grover RF. Lung mast cell hyperplasia and pulmonary histamine-forming capacity in hypoxic rats. *Am J Physiol* 1974; 226: 178-184.
14. Tucker A, McMurtry IF, Alexander AF, Reeves JT, Grover RF. Lung mast cell density and distribution in chronically hypoxic animals. *J Appl Physiol* 1977; 42: 174-178.
15. Maclean MR, Dempsey Y. Serotonin and pulmonary hypertension - from bench to bedside? *Curr Opin Pharmacol* 2009; 9: 281-286.
16. Ferreira AJ, Shenoy V, Yamazato Y, Sriramula S, Francis J, Yuan L, Castellano RK, Ostrov DA, Oh SP, Katovich MJ, Raizada MK. Evidence for angiotensin-converting enzyme 2 as a therapeutic target for the prevention of pulmonary hypertension. *Am J Respir Crit Care Med* 2009; 179: 1048-1054.
17. Miyazaki M, Takai S, Jin D, Muramatsu M. Pathological roles of angiotensin II produced by mast cell chymase and the effects of chymase inhibition in animal models. *Pharmacol Ther* 2006; 112: 668-676.
18. Tchougounova E, Lundequist A, Fajardo I, Winberg JO, Abrink M, Pejler G. A key role for mast cell chymase in the activation of pro-matrix metalloproteinase-9 and pro-matrix metalloproteinase-2. *J Biol Chem* 2005; 280: 9291-9296.
19. Maxova H, Novotna J, Vajner L, Tomasova H, Vytasek R, Vizek M, Bacakova L, Valouskova V, Eliasova T, Herget J. In vitro hypoxia increases production of matrix metalloproteinases and tryptase in isolated rat lung mast cells. *Physiol Res* 2008; 57: 903-910.
20. Okayama Y, Ra C, Saito H. Role of mast cells in airway remodeling. *Curr Opin Immunol* 2007; 19: 687-693.

21. Savale L, Tu L, Rideau D, Izziki M, Maitre B, Adnot S, Eddahibi S. Impact of interleukin-6 on hypoxia-induced pulmonary hypertension and lung inflammation in mice. *Respir Res* 2009; 10: 6.
22. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am J Respir Crit Care Med* 1995; 151: 1628-1631.
23. Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. *Immunol Rev* 2009; 228: 149-169.
24. Klein M, Schermuly RT, Ellinghaus P, Milting H, Riedl B, Nikolova S, Pullamsetti SS, Weissmann N, Dony E, Savai R, Ghofrani HA, Grimminger F, Busch AE, Schafer S. Combined tyrosine and serine/threonine kinase inhibition by sorafenib prevents progression of experimental pulmonary hypertension and myocardial remodeling. *Circulation* 2008; 118: 2081-2090.
25. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, Grimminger F. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 2005; 115: 2811-2821.
26. Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM, Zarrinkar PP. A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* 2008; 26: 127-132.
27. Castillo JG, Oehling A, Gamboa PM. Mechanism of ketotifen action in hypersensitivity reactions. Its effect on cellular enzymatic activities. *J Invest Allergol Clin Immunol* 1991; 1: 315-323.
28. Lowe DA, Richardson BP. Effects of cyproheptadine, ketotifen and sodium nitroprusside on mechanical activity and calcium uptake in guinea pig taenia coli in vitro. *Respiration* 1980; 39 Suppl 1: 44-46.
29. Davie NJ, Crossno JT, Frid MG, Hofmeister SE, Reeves JT, Hyde DM, Carpenter TC, Brunetti JA, McNiece IK, Stenmark KR. Hypoxia-induced pulmonary artery adventitial remodeling and neovascularization: contribution of progenitor cells. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L668-L678.

30. Miettinen M, Lasota J. KIT (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. *Appl Immunohistochem Mol Morphol* 2005; 13: 205-220.
31. Banasova A, Maxova H, Hampl V, Vizek M, Povysilova V, Novotna J, Vajnerova O, Hnilickova O, Herget J. Prevention of mast cell degranulation by disodium cromoglycate attenuates the development of hypoxic pulmonary hypertension in rats exposed to chronic hypoxia. 2008; *Respiration* 76: 102-107.
32. Zhu YJ, Kradin R, Brandstetter RD, Staton G, Moss J, Hales CA. Hypoxic pulmonary hypertension in the mast cell-deficient mouse. *J Appl Physiol* 1983; 54: 680-686.

Figure Legends

Table 1. Selected differentially regulated genes in PH owing to left heart disease. Selected genes out of a total of 120 genes with differential expression in lungs of rats with supracoronary aortic banding as compared to sham operated controls. Differential expression was defined as ≥ 4 fold change with a $p < 0.05$. Data from $n=3$ animals each. * \uparrow/\downarrow symbolize up- and downregulation in banded as compared to control rats; † fold change is expressed as ratio of gene expression in banded/control rats; PAI-1, plasminogen activator inhibitor-1 (serpinE1); ET_B receptor, endothelin-1 type B receptor. Mast cell specific genes are highlighted.

TABLE 1:

Category	Gene	\uparrow/\downarrow^*	Fold change †	p
	interleukin 1 alpha	↓	0.17	0.009
	Il-1 receptor antagonist	↑	4.43	0.025
	Il-8 receptor, alpha	↑	8.65	0.003
inflammation/ immune response	oxLDL receptor 1	↑	5.65	0.025
	mast cell protease 1	↑	10.31	0.030
	mast cell peptidase 2	↑	4.53	0.003
	tryptase	↑	4.12	0.013
	chymase 1	↑	6.81	0.001
complement system	complement factor 1	↑	5.71	<0.001
	complement component 6	↑	31.77	0.020
coagulation system	PAI-1	↑	6.47	<0.001
vascular regulation	ET _B receptor	↓	0.23	0.049
	heme oxygenase 1	↑	6.98	0.016
	endothelial lipase	↓	0.17	0.019
extracellular matrix composition	vitronectin	↓	0.05	0.007
	procollagen type XV	↑	4.42	0.040
	elastin	↑	4.58	0.030
developmental pathways	semaphorin 6A	↓	0.12	0.014

Figure 1. Lung mast cell accumulation in rats with supracoronary aortic banding. **A:** Volcano blot from whole rat genome microarray analyses (28,000 genes in total) shows differential gene expression in lung homogenates of rats with aortic banding as compared to controls (n=3 each). Red spots depict 120 genes that are significantly ($p < 0.05$; $-\text{Log}_{10} > 1.3$) and more than 4 fold up- ($\text{Log}_2 > 2$) or downregulated ($\text{Log}_2 < -2$) in banded as compared to control rats. **B:** Heatmap depicts expression pattern of the 120 differentially regulated genes from 3 lungs of rats with aortic banding (AO) and 3 control lungs. Color code (red up-, green downregulation) shows individual gene expression relative to mean gene expression of the whole array. Annotation according to NCBI gene symbols. **C:** Dendrogram of the gene ontology (GO) cluster "immune system process" with respective GO terms and accession numbers. Within the entire microarray, differential regulation was most pronounced in the GO class "mast cell activation". **D:** Bar graphs show upregulation of mast cell chymase and mast cell peptidase 2 in aortic banding as compared to control rats. Data from real time RT-PCR of lung homogenates were quantified in comparison to standard curves produced under the same cycling conditions and normalized to GAPDH expression from the same experiment. **E:** Bar graphs and representative Western blots from lung homogenate show increased protein expression of mast cell chymase and mast cell peptidase 2 in lungs from aortic banding rats as compared to controls, tubulin- α serves as loading control. Data from n=3 each; * $p < 0.05$ vs. control.

Figure 1

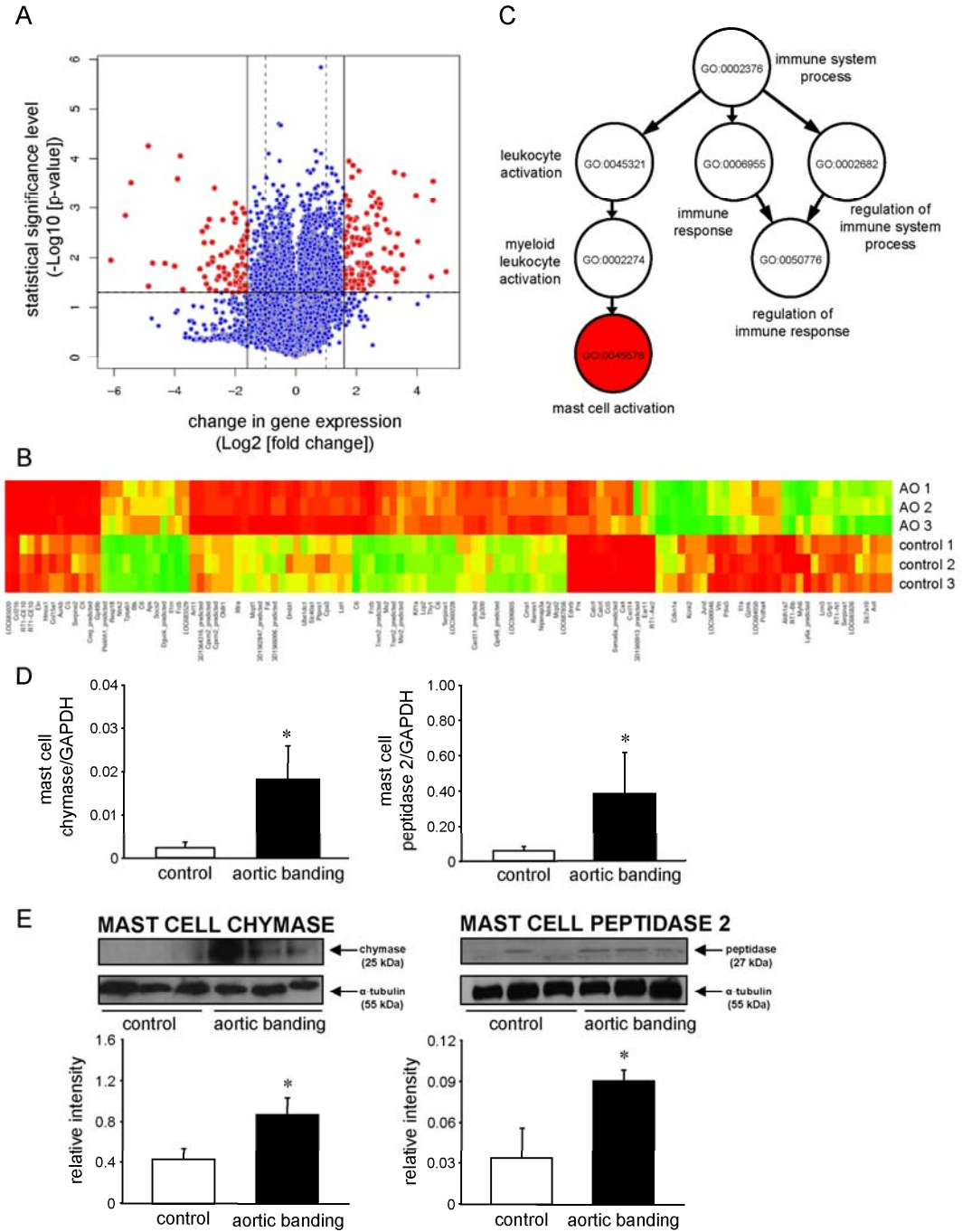


Figure 2. Effect of the mast cell stabilizer ketotifen on hemodynamics and right ventricular dysfunction in PH owing to left heart disease. **A:** Bar graphs PAP, PVR and right ventricular (RV) weight relative to body weight (bw) in controls, banded rats and ketotifen-treated banded rats. Data from n=6 each. **B:** Representative echocardiographic 2D-images show left (LV) and right (RV) ventricle in end-diastole in control, banded, and ketotifen-treated banded rats. Note marked dilation of the right ventricle in banded rats, which is attenuated by ketotifen. Replicated in n=10 each. **C:** Bar graphs show right (RV) and left (LV) ventricular end-diastolic area, and tricuspid annular plane systolic excursion (TAPSE) in control, banded, and ketotifen-treated banded rats. Data from n=10 each; *p<0.05 vs. control, #p<0.05 vs. aortic banding.

Figure 2

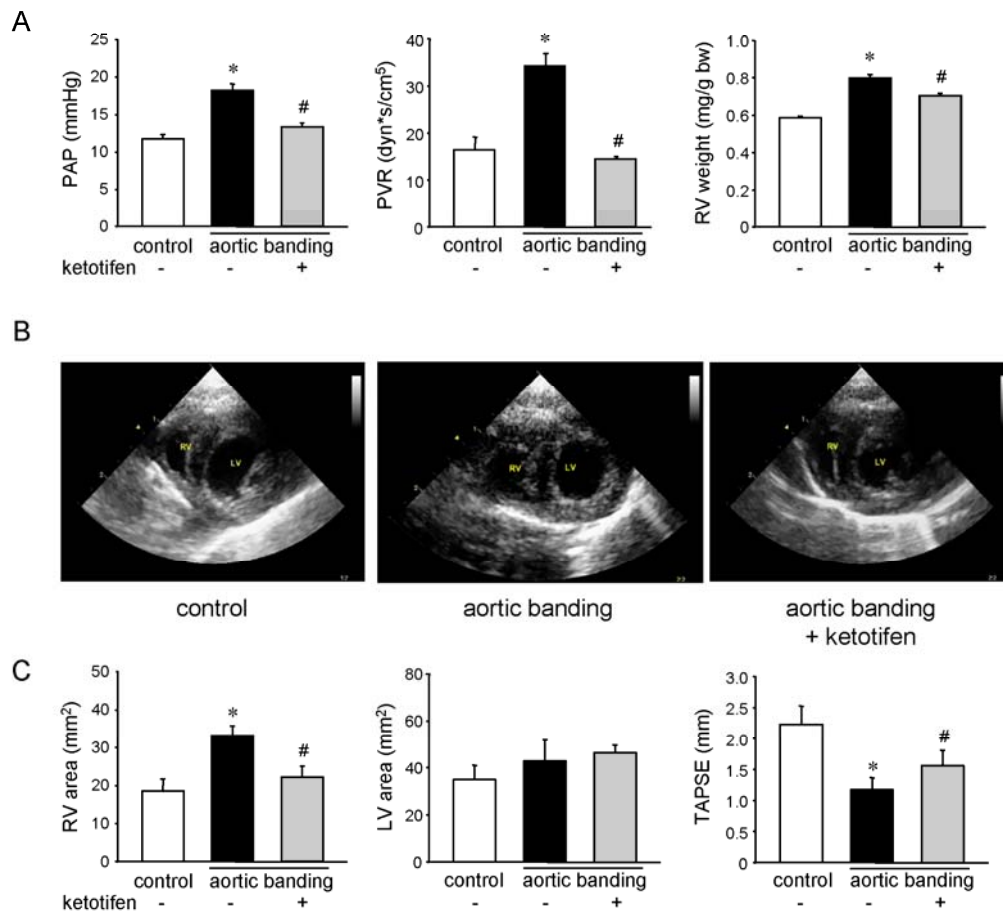


Figure 3. Effect of the mast cell stabilizer ketotifen on lung vascular remodeling and pulmonary mast cells in PH owing to left heart disease. **A:** Representative images of HE-stained lung sections from control, banded, and ketotifen-treated banded rats. Medial thickening in lung vessels from banded rats is largely abrogated by ketotifen. Replicated in n=5 each. **B:** Bar graphs show medial wall thickness for pulmonary arterioles sized 25–50 μm ; 50–100 μm ; and >100 μm in diameter in lungs from control, banded, and ketotifen-treated banded rats. **C:** Bar graphs show the degree of muscularization and internal lumen area of pulmonary arterioles of 20–50 μm diameter in lungs from control, banded, and ketotifen-treated banded rats. Degree of muscularization is expressed as proportion of non-, partially, or fully muscularized pulmonary arterioles in percentage of total pulmonary arteriolar cross section. Data from n=5 each. **D:** Bar graphs show mast cell density in lung histological sections, index of granulation and number of perivascular mast cells surrounding pulmonary vessels sized 20–50 μm ; 50–150 μm ; and >150 μm in diameter in lungs from control, banded, and ketotifen-treated banded rats. Data from n=8 each, *p<0.05 vs. control, #p<0.05 vs. aortic banding.

Figure 3

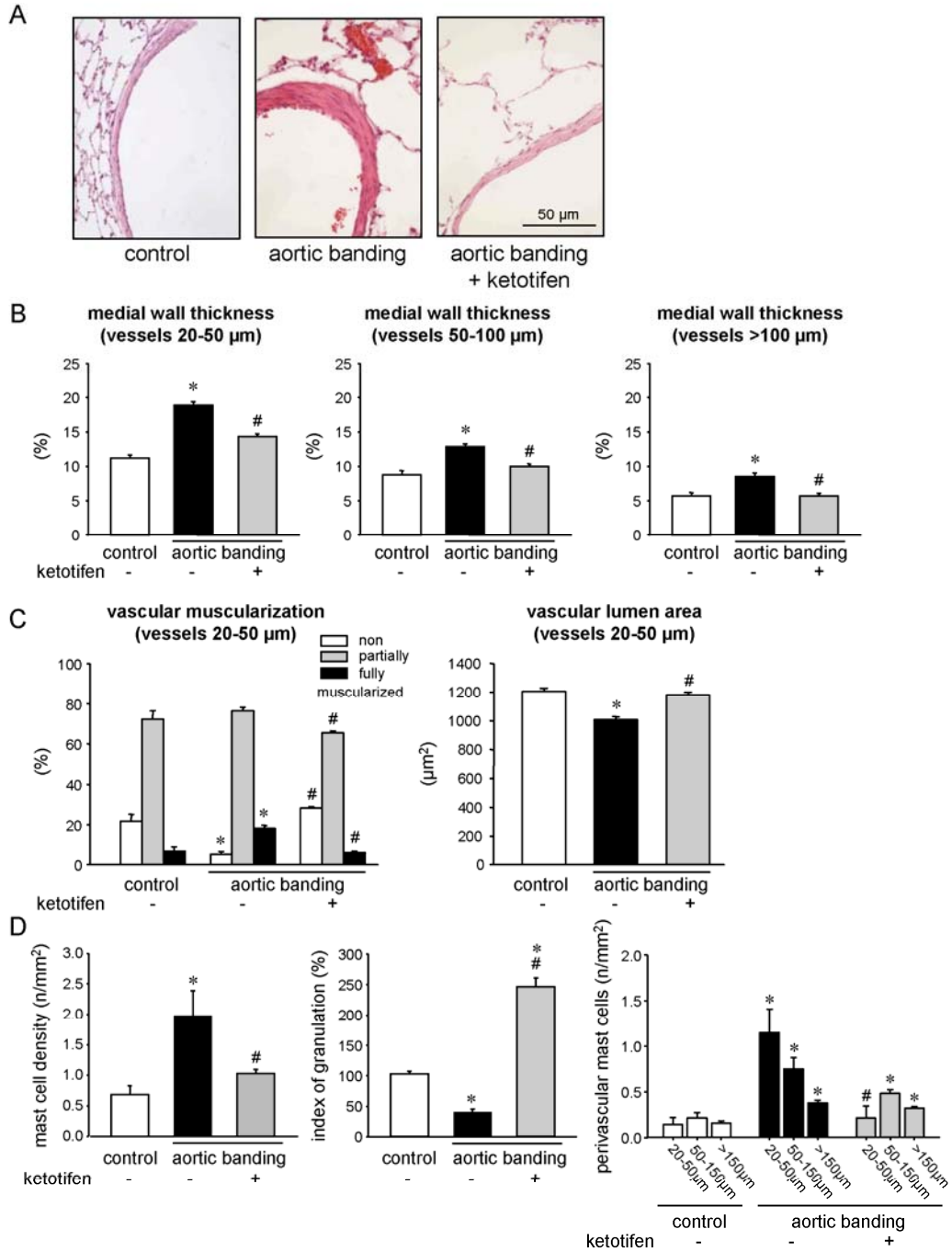


Figure 4. Vascular remodelling and PH owing to left heart disease in mast cell deficient *Ws/Ws* rats. **A:** Bar graphs show PAP, PVR and RV weight in control wild type (WT) rats, banded WT rats, and banded *Ws/Ws* rats. Data from n=6 each. **B:** Bar graphs show medial wall thickness for pulmonary arterioles sized 25–50 μm ; 50–100 μm ; and >100 μm in diameter in lungs from control WT, banded WT, and banded *Ws/Ws* rats. **C:** Bar graphs show the degree of muscularization and internal lumen area of pulmonary arterioles of 20–50 μm diameter in lungs from control WT, banded WT, and banded *Ws/Ws* rats. Data from n=5 each. **D:** Bar graphs show mast cell density in lung histological sections from control WT, banded WT, and banded *Ws/Ws* rats, and index of granulation and number of perivascular mast cells surrounding pulmonary vessels sized 20–50 μm ; 50–150 μm ; and >150 μm in diameter in lungs from control WT and banded WT rats. Data from n=8 each, *p<0.05 vs. control WT, #p<0.05 vs. aortic banding WT.

Figure 4

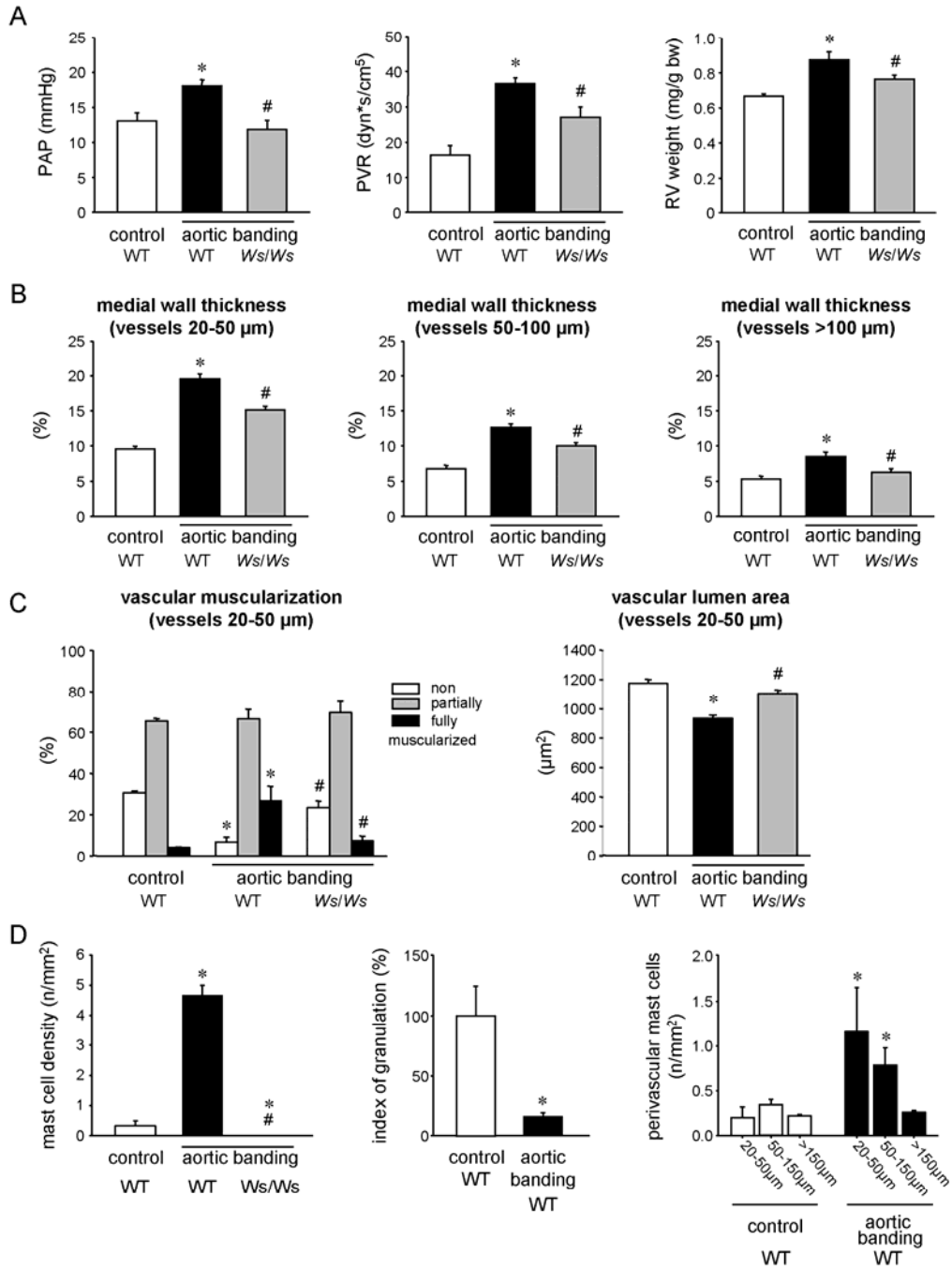


Figure 5. Effect of the mast cell stabilizer ketotifen in monocrotaline-induced PH. **A.** Bar graphs show PAP, PVR and RV weight in control rats, monocrotaline (MCT) treated rats, and ketotifen-treated MCT rats. Data from n=6 each. **B:** Bar graphs show right (RV) and left (LV) ventricular end-diastolic area and medial wall thickness of pulmonary arteries in control, MCT, and ketotifen-treated MCT rats. Data from n=10 each. **C:** Bar graphs show mast cell density in lung histological sections, index of granulation and number of perivascular mast cells surrounding pulmonary vessels sized 20–50 μm ; 50–150 μm ; and >150 μm in diameter in lungs from control, MCT, and ketotifen-treated MCT rats. Data from n=8 each, *p<0.05 vs. control, #p<0.05 vs. MCT.

Figure 5

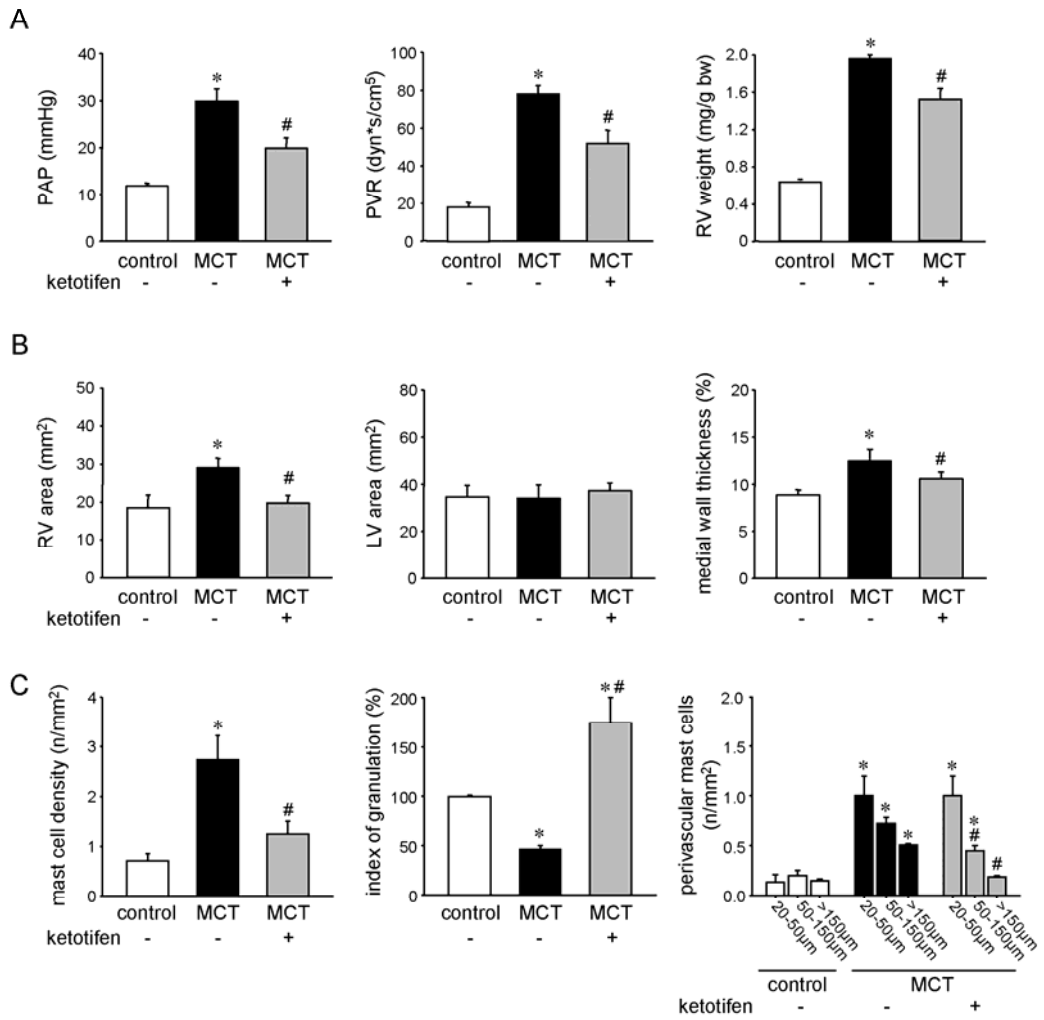


Figure 6. Vascular remodelling and PH in monocrotaline-treated mast cell deficient *Ws/Ws* rats. **A.** Bar graphs show PAP, PVR and RV weight in control wild type (WT) rats, monocrotaline (MCT) treated WT rats, and MCT treated *Ws/Ws* rats. Data from n=6 each. **B:** Bar graphs show right ventricular (RV) end-diastolic area, tricuspid annular plane systolic excursion (TAPSE) and medial wall thickness of pulmonary arteries in control WT, MCT WT, and MCT *Ws/Ws* rats. Data from n=10 each; *p<0.05 vs. control WT, #p<0.05 vs. MCT WT.

Figure 6

