The angiopietin-1–Tie2 pathway prevents rather than promotes pulmonary arterial hypertension in transgenic mice

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The role of the angiopoietin-1 (Ang1)–Tie2 pathway in the pathogenesis of pulmonary arterial hypertension (PAH) is controversial. Although Ang1 is well known to prevent endothelial activation and injury in systemic vascular beds, this pathway has been suggested to mediate pulmonary vascular remodeling in PAH. Therefore, we used transgenic models to determine the effect of increased or decreased Tie2 activity on the development of PAH. We now report modest spontaneous elevation in right ventricular systolic pressure in *Tie2*-deficient mice (*Tie2*$^{+/-}$) compared with wild-type (WT) littermate controls, which was exacerbated upon chronic exposure to the clinically relevant PAH triggers, serotonin (5-HT) or interleukin-6 (IL-6). Moreover, overexpression of Ang1 in transgenic mice had no deleterious effect on pulmonary hemodynamics and, if anything, blunted the response to 5-HT. Exposure to 5-HT or IL-6 also decreased lung Ang1 expression, further reducing Tie2 activity and inducing pulmonary apoptosis in the *Tie2*$^{+/-}$ group only. Similarly, cultured pulmonary artery endothelial cells subjected to *Tie2* silencing demonstrated increased susceptibility to apoptosis after 5-HT treatment. Finally, treatment of *Tie2*-deficient mice with Z-VAD, a pan-caspase inhibitor, prevented the pulmonary hypertensive response to 5-HT. Thus, these findings firmly establish that endothelial survival signaling via the Ang1–Tie2 pathway is protective in PAH.

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Abbreviations used: 5-HT, serotonin; Ang, angiopoietin; BT, binary transgenic; EC, endothelial cell; eNOS, endothelial NO synthase; NBT, non-BT; PAH, pulmonary arterial hypertension; PI, propidium iodide; RV, right ventricle; RVSP, right ventricular systolic pressure; SBP, systolic blood pressure; sIL-6R, soluble IL-6R; SMC, smooth muscle cell; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.

The pathogenesis of pulmonary arterial hypertension (PAH) is complex and poorly understood; however, pulmonary arterial endothelial cell (EC) injury is considered to be a critical early mechanism in the initiation and progression of disease (Budhiraja et al., 2004; Voelkel and Cool, 2004; Song et al., 2008). The distal pulmonary arteriolar bed normally lacks muscularization at the level of the intraacinar arteriole, which essentially consists of an EC tube with limited matrix support (Tuder et al., 2001) exposed directly to the environment through inspired air. Therefore, we have hypothesized that these uniquely fragile precapillary structures may be particularly susceptible to damage upon exposure to environmental stress, especially in the context of genetic predisposition, and that EC apoptosis contributes to the functional and structural abnormalities of the pulmonary microvasculature characteristic of this disease.

Tie2 belongs to the family of endothelial-selective receptor tyrosine kinases and is essential for both embryonic and postnatal angiogenesis (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996; Ward and Dumont, 2002). Angiopoietin-1 (Ang1) and Ang2 are the principle Tie2 ligands. Ang1 is a Tie2 agonist and functions as a homeostatic factor in the postnatal vasculature, protecting against EC inflammation and apoptosis mainly through activation of the Akt pathway (Papapetropoulos et al., 2000). In contrast, Ang2 was first identified as an endogenous antagonist to Tie2 (Maisonpierre et al.,

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1997) but is now recognized to have context-dependent agonistic properties, and has been reported to activate the Tie2 receptor at high concentration (Kim et al., 2000) or during prolonged incubation (Teichert-Kuliszewska et al., 2001).

Despite considerable evidence supporting a protective action for the Ang1–Tie2 pathway in systemic blood vessels (Chen and Stinnett, 2008; Lai et al., 2008; Lee et al., 2008), the role of this system in the pulmonary microvasculature, particularly in PAH, remains highly controversial. Thistlethwaite et al. reported remarkable increases in Ang1 expression and Tie2 activity in the lungs of PAH patients compared with control lung samples, in which both were nearly undetectable (Thistlethwaite et al., 2001; Du et al., 2003). However, other groups have found high basal levels of Ang1 expression and Tie2 activity in the normal human lung (Kugathasan et al., 2005; Eddahibi et al., 2006); indeed, the lung has been reported to exhibit the highest levels of Ang1–Tie2 expression and activity of all organs (Wong et al., 1997; Witzenbichler et al., 1998). Nonetheless, the Thistlethwaite group has also reported that overexpression of Ang1 by means of transfection with adenoviral (Sullivan et al., 2003) or adeno-associated viral vectors (Chu et al., 2004) produced PAH in rats. Again, these findings are in conflict with other reports demonstrating that nonviral *Ang1* gene transfer reduced right ventricular systolic pressure (RVSP) and vascular remodeling in both the monocrotaline (Zhao et al., 2003) and chronic hypoxia (Kugathasan et al., 2005) models of PAH, even improving survival in the former. These discrepancies may be related to confounding influences of viral vectors that were used for transfection, because these may have proinflammatory effects, or surgical manipulation, in particular the clamping of delicate pulmonary veins that was performed to enhance pulmonary gene transfer (Sullivan et al., 2003; Chu et al., 2004).

Therefore, in the present study we relied on transgenic approaches to study the effects of both loss or gain of function of the Ang1–Tie2 pathway on pulmonary hemodynamics and remodeling. We now report that *Tie2* heterozygous deficient mice showed mild spontaneous increases in RVSP and an exaggerated pulmonary hypertensive response to chronic exposure to serotonin (5-HT) or IL-6. This was associated with decreased lung Ang1 levels and Tie2 receptor activation, and increased peripheral lung apoptosis in *Tie2*-deficient mice only. Moreover, the inhibition of apoptosis with a pan-caspase inhibitor, Z-VAD, prevented PAH in response to 5-HT in $Tie2^{+/-}$ animals, whereas overexpression of Ang1 using a conditional transgenic model did not result in PAH and, if anything, blunted the pulmonary hypertensive response to 5-HT exposure. Therefore, the evidence from these transgenic models supports the view that the Ang1–Tie2 pathway acts to protect against the development of PAH, largely by preventing apoptosis and early lung microvascular loss.

RESULTS

Phenotypic characterization of WT and $Tie2^{+/-}$ mice

Western blot analysis of lung Tie2 and endothelial NO synthase (eNOS) protein revealed a reduction of ~50% in expression levels in $Tie2^{+/-}$ compared with WT mice ($P < 0.01$ and 0.05, respectively; Fig. 1, A and B). Interestingly, basal protein levels of Ang1 in the lung were slightly increased in $Tie2^{+/-}$ compared with WT mice ($P < 0.01$), whereas Ang2 levels were reduced ($P < 0.01$), resulting in a more than twofold increase in the ratio of lung Ang1 to Ang2 levels ($P < 0.05$; Fig. 1 C). Under basal conditions, there was a modest but significant difference in RVSP between WT and $Tie2^{+/-}$ mice ($P < 0.05$), with 13% of the $Tie2^{+/-}$ mice demonstrating a RVSP measurement >37 mmHg (two standard deviations above mean RVSP of WT mice; χ^2, $P < 0.05$ compared with WT mice; Fig. 2 A); however, no significant change in baseline systemic systolic blood pressure (SBP; Fig. 2 B) or heart rate (unpublished data) was observed. A marked reduction in microvascular perfusion in the lungs of $Tie2^{+/-}$ compared with WT mice was revealed after fluorescent microangiography (Fig. 2 C). Furthermore, this perfusion abnormality was more pronounced in mice with higher RVSPs. To further characterize their susceptibility to PAH, WT and $Tie2^{+/-}$ mice were subjected to 1 wk of hypoxic exposure (8–10% O_2). Chronic hypoxia resulted in a significant increase in RVSP and right ventricle (RV) hypertrophy ($P < 0.05$ and 0.01, respectively, compared with normoxic controls), but there was no significant difference between the hypoxic WT and $Tie2^{+/-}$ mice observed (Fig. S1).

Effect of 5-HT or IL-6 on RVSP and RV hypertrophy

To determine the effect of well-known pulmonary hypertensive stimuli, RVSP (Fig. 3 A) and RV hypertrophy (Fig. 3 B) were assessed in WT and $Tie2^{+/-}$ mice after 1 wk of 5-HT infusion and 1 or 2 wk of IL-6 delivery. Treatment with 5-HT had no significant effect on RVSP in WT mice, although there was a trend ($P < 0.1$) toward an increase. However, 5-HT–treated *Tie2*-deficient mice exhibited a substantial increase in RVSP compared with their respective saline-treated controls (40 ± 4 vs. 25 ± 1 mmHg; $P < 0.01$). This increase was also significantly greater than that seen in WT mice exposed to 5-HT (27 ± 2 mmHg; $P < 0.05$). Although no significant effect on RVSP was observed in any group after 1 wk of IL-6 delivery, 2 wk of exposure to IL-6 induced a significant elevation in RVSP in $Tie2^{+/-}$ mice compared with IL-6–treated WT mice (31 ± 3 vs. 22 ± 1 mmHg; $P < 0.05$). A trend ($P < 0.1$) toward an increase in RVSP was also observed in $Tie2^{+/-}$ mice compared with their saline-treated controls. No significant change in RV hypertrophy was observed in either the WT or $Tie2^{+/-}$ mice during the short period of treatment with 5-HT. However, a significant increase in RV hypertrophy was observed in $Tie2^{+/-}$ compared with WT mice after 2 wk of exposure to IL-6 ($P < 0.05$).

Effect of 5-HT or IL-6 on Ang1 level and Tie2 activity

Western blot analysis was performed on lung homogenates from WT and $Tie2^{+/-}$ mice to determine whether treatment with 5-HT or IL-6 altered endogenous Ang1 levels (Fig. 4, A and B). After 1 wk of exposure to 5-HT or IL-6, Ang1 levels were significantly and similarly decreased in WT and $Tie2^{+/-}$ mice compared with their respective saline-treated

control groups ($P < 0.05$). To further establish the association between 5-HT and Ang1, human pulmonary artery smooth muscle cells (SMCs) were exposed for 24 h to 5-HT (10^{-8}–10^{-5} M) or a combination of equal concentrations of IL-6 and a soluble IL-6R (sIL-6R; 10, 50, and 100 ng/ml), because past studies (Modur et al., 1997; Ammit et al., 2007) have demonstrated that the membrane-bound receptor may not be expressed in SMCs under in vitro conditions and that the soluble receptor is required to allow for IL-6 trans-signaling (Fig. S2). Ang1 secretion, as determined by ELISA performed on conditioned media, was reduced to ~60% in response to 5-HT or IL-6/sIL-6R ($P < 0.01$; Fig. 4, C and D). To investigate the implications of these observations on in vivo Tie2 activity, Western blot analysis of phosphorylated Tie2 was performed on lung homogenates (Fig. 5, A and B). Although in WT mice no change in Tie2 receptor activation was observed after 5-HT infusion or IL-6 injections, *Tie2*$^{+/-}$ mice displayed a significant decrease in lung Tie2 receptor activation compared with their saline-treated groups, in parallel to the decreases in Ang1 levels demonstrated in Fig. 4 (A and B) ($P < 0.05$). Because baseline levels of eNOS were significantly down-regulated in *Tie2*$^{+/-}$ mice (Fig. 1 B), we examined eNOS protein expression in WT and *Tie2*$^{+/-}$ mice after 5-HT infusion (Fig. S3). Interestingly, although there was no significant reduction in lung eNOS protein levels after 5-HT exposure, a trend toward a decrease ($P < 0.1$) was observed in the *Tie2*-deficient group.

Cell-death analysis

No terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining or propidium iodide (PI) uptake was detected in saline-treated WT or *Tie2*$^{+/-}$ mice (not depicted). In contrast, substantial TUNEL positivity was seen after 1 wk of exposure to 5-HT in *Tie2*-deficient but not in WT mice (7 vs. 0%; $P < 0.01$; Fig. 6 A). This increase in apoptosis was confirmed by in vivo perfusion of the pulmonary circulation with PI, which only stains the nuclei of nonviable cells. Again, only *Tie2*$^{+/-}$ mice exposed to 5-HT demonstrated an increased uptake of PI (Fig. S4). Similarly, after 1 wk of exposure to IL-6, only *Tie2*-deficient mice

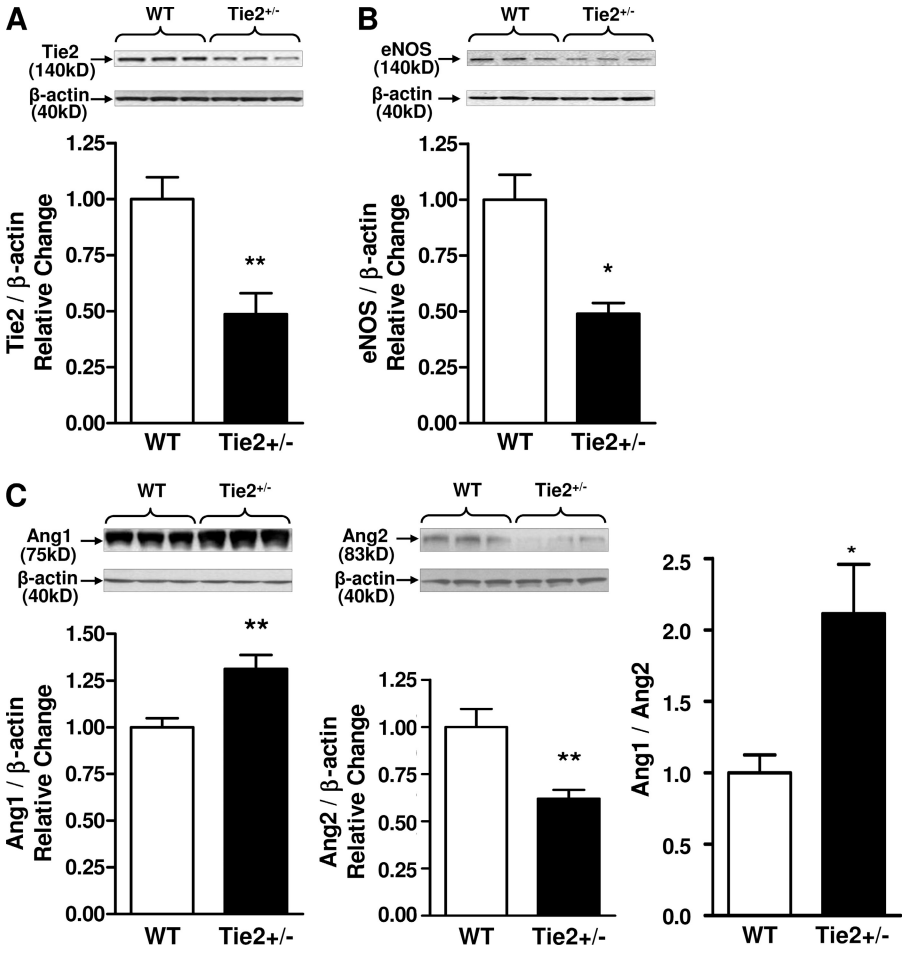

Figure 1. Characterization of lung protein expression in WT and *Tie2*$^{+/-}$ mice under basal conditions. (A and B) Decreased lung Tie2 (A; $n = 5$ mice per group) and eNOS protein levels (B; $n = 3$ mice per group) in *Tie2*$^{+/-}$ compared with WT mice. (C) Increased lung Ang1 and decreased lung Ang2 protein levels, and increased ratio of lung Ang1 to Ang2 protein levels in *Tie2*$^{+/-}$ compared with WT mice ($n = 5$ mice per group). Results in A and C are from two independent experiments, and results in B are from one experiment. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$ versus WT.

demonstrated TUNEL staining within peripheral regions of the lung (6%), whereas no staining was seen in WT mice ($P < 0.01$; Fig. 6 B).

Effect of reduced Tie2 expression and 5-HT on pulmonary artery EC survival

To further define the effect of decreased Tie2 receptor expression and signaling on EC survival in response to 5-HT, *Tie2* was knocked down in human pulmonary artery ECs using specific silencing siRNA. Transfection with specific siRNA reduced Tie2 protein expression by ~50% by Western analysis, compared with control, scrambled siRNA–treated ECs ($P < 0.01$; Fig. 7 A). Furthermore, *Tie2* silencing significantly enhanced EC apoptosis induced by 10 μM 5-HT cultured in low serum (0.2% fetal bovine serum) compared with cells treated with scrambled siRNA ($P < 0.01$; Fig. 7 B).

Effect of 5-HT or IL-6 on pulmonary vascular remodeling

Exposure to 5-HT for 1 wk did not result in any differences in the muscularization of pulmonary vessels <50 μm in diameter in either WT or *Tie2*-deficient mice (unpublished data). However, $Tie2^{+/-}$ mice exposed to 2 wk of IL-6 demonstrated a significant increase in muscularization compared with saline-treated $Tie2^{+/-}$ and IL-6-treated WT mice ($P < 0.01$ for both comparisons; Fig. 8, A and B). Preliminary studies demonstrated no overt phenotypic differences in extracellular matrix composition between WT and $Tie2^{+/-}$ mice after exposure to 5-HT or IL-6 (Fig. S5).

Lack of PAH in Ang1 transgenic mice

Protein expression of the human Ang1 transgene was detected in the mouse plasma of binary transgenic (BT) mice after induction by doxycycline withdrawal (Fig. S6). To define the effects of Ang1 overexpression on the development of PAH, RVSP and RV hypertrophy were determined in 11–12-wk-old *Ang1* BT mice and non-BT (NBT) littermate controls that had been released from doxycycline suppression at 3 wk of age, and then received infusions of 5-HT or saline for 1 wk before end-study assessments. As presented in Fig. 9 A, there were no significant differences in RVSP between *Ang1* BT and NBT mice receiving either saline or 5-HT infusions; if anything, RVSP tended to be reduced in BT mice, but this was not significant. As well, no differences in RV hypertrophy were observed between the groups after 1 wk of saline or 5-HT treatment (Fig. 9 B).

Effect of caspase inhibition

To further establish the role of EC apoptosis in the exaggerated pulmonary hypertensive response to 5-HT in vivo, we treated WT or *Tie2*-deficient animals with a pan-caspase inhibitor, Z-VAD. In WT mice, Z-VAD had no effect, whereas in $Tie2^{+/-}$ mice, this treatment completely prevented the

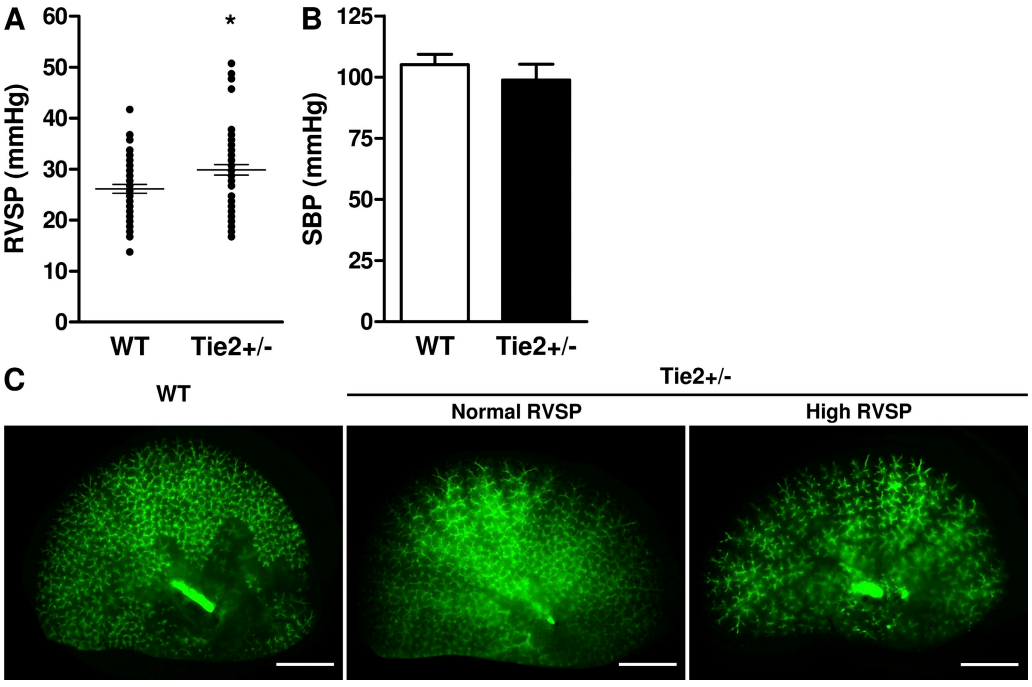

Figure 2. Hemodynamic analysis and pulmonary fluorescent microangiography in WT and $Tie2^{+/-}$ mice under basal conditions. (A) Elevation in baseline RVSP measurements in $Tie2^{+/-}$ ($n = 55$) compared with WT ($n = 60$) mice. (B) No significant difference in systemic SBP between WT and $Tie2^{+/-}$ mice was observed ($n = 5$ mice per group). (C) Fluorescent microangiography (whole top right lung lobe) illustrating decreased microcirculatory perfusion in baseline $Tie2^{+/-}$ compared with WT mice; perfusion is further reduced in mice with higher RVSP measurement ($n = 5$ mice per group). Results in A are from 10 independent experiments, each with similar proportions of WT and $Tie2^{+/-}$ mice; results in B are from 2 independent experiments; and representative photomicrographs in C are from three independent experiments. Data are presented as means ± SEM. *, $P < 0.05$ versus WT. Bars, 0.25 cm.

exaggerated increase in RVSP induced by 5-HT ($P < 0.01$; Fig. 10 A). In addition, no significant change in RV hypertrophy was present at 1 wk (Fig. 10 B). Furthermore, no TUNEL-positive cells were detected in the lungs of *Tie2*$^{+/-}$ mice receiving both 5-HT and Z-VAD (not depicted).

DISCUSSION

In this study, we examined whether alterations in Tie2 activity would protect or predispose to the development of PAH using loss- and gain-of-function transgenic models. Ang1 overexpression by itself did not induce PAH, and *Tie2* haploinsufficiency resulted in spontaneous increases in RVSP in a small proportion of mice under basal conditions. Moreover chronic treatment of *Tie2*-deficient mice with 5-HT or IL-6 unmasked significant elevation in pulmonary arterial pressures compared with WT animals. These interventions also reduced lung Ang1 levels and Tie2 activity, and induced EC apoptosis both in vivo and in vitro, but only in the context of *Tie2* deficiency. As well, inhibition of apoptosis with Z-VAD completely prevented the pulmonary hypertensive response to 5-HT in *Tie2*$^{+/-}$ mice. These data strongly suggest that the Ang1–Tie2 pathway protects against PAH, in large part by promoting EC survival under conditions of serotonergic or inflammatory stress, both of which have been strongly implicated in the pathogenesis of this disease.

The distal pulmonary arteriolar bed is unique in that it consists of little more than an endothelial tube with scant matrix and few, if any, supporting mural cells. Thus, the ECs of the precapillary arterioles may be particularly vulnerable to injurious stimuli, and thereby more dependent on survival signaling, such as that afforded by Ang1 and vascular endothelial growth factor, to maintain lung vascular homeostasis. This is consistent with previous observations that inhibiting vascular endothelial growth factor signaling can potentiate experimental PAH (Taraseviciene-Stewart et al., 2001), whereas overexpression of angiogenic factors can be protective (Janssens et al., 1996; Campbell et al., 2001; Zhao et al., 2003; Kugathasan et al., 2005; Zhao et al., 2005). Moreover, we hypothesize that the functional consequences of even low levels of EC apoptosis may be particularly important for this region, because there is a finite possibility that this could disrupt the functional continuity between the arteriolar and capillary circulations, effectively excluding an "arteriolar-capillary unit" from the efficient "low pressure" pulmonary circulation. If this occurs repeatedly over time throughout the lung microvasculature, this would result in progressive increases in pulmonary vascular resistance.

Tie2 is an endothelial-selective receptor tyrosine kinase that is essential for vascular growth and remodeling. A null mutation of *Tie2* results in embryonic lethality at E9.5–12.5 with marked vascular abnormalities, including reduced vascular

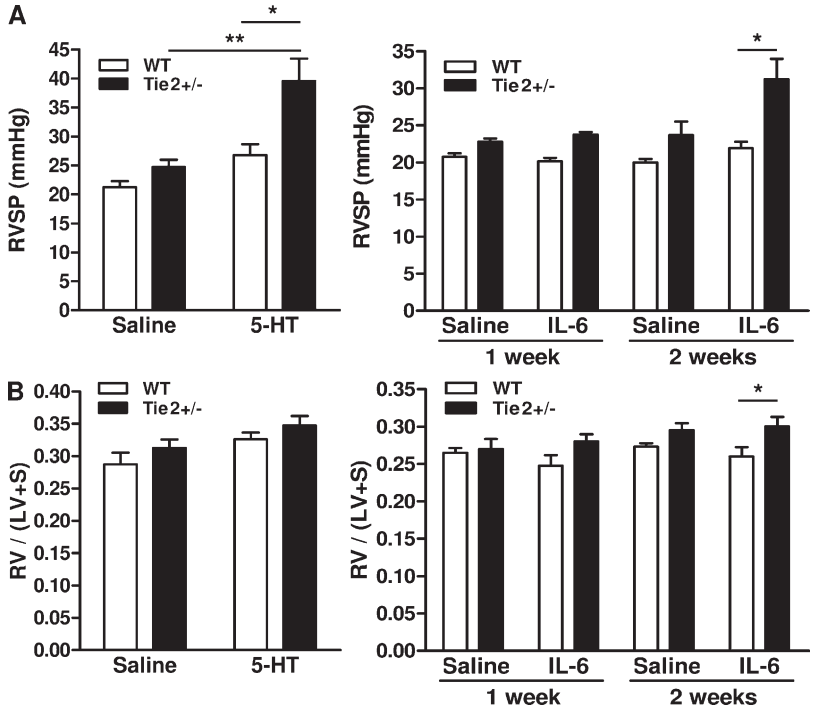

Figure 3. Effect of 5-HT or IL-6 on RVSP and RV hypertrophy. (A) Increased RVSP in *Tie2*$^{+/-}$ mice exposed to 5 nmol/h 5-HT for 1 wk compared with WT mice and compared with saline-treated *Tie2*$^{+/-}$ mice (n = 12–15 mice per group). Increased RVSP in *Tie2*$^{+/-}$ mice exposed to 200 ng/kg/d IL-6 for 2 wk compared with WT mice (n = 9–13 mice per group). (B) No change in RV hypertrophy, assessed by evaluating the mass ratio of the RV to the left ventricle plus septum (LV+S), in WT or *Tie2*$^{+/-}$ mice exposed to 5 nmol/h 5-HT for 1 wk compared with saline-treated groups (n = 12–15 mice per group). Increased RV hypertrophy in *Tie2*$^{+/-}$ mice exposed to IL-6 for 2 wk compared with WT mice (n = 9–13 mice per group). Results are from three independent experiments. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$ as indicated.

complexity, increased EC apoptosis, and an absence in mural cell recruitment (Davis et al., 1996). Furthermore, Ang1 is highly expressed by SMCs and pericytes in the postnatal vasculature, and promotes vascular stabilization and maintenance of EC quiescence (Maisonpierre et al., 1997). Of interest, the lung exhibits some of the highest levels of basal Ang1 expression of any organ in the body (Wong et al., 1997). In addition, *Tie2*-deficient mice exhibited a marked down-regulation of lung eNOS protein levels, and any reduction in the release of the vascular protective and EC survival factor, NO, could contribute to EC dysfunction and loss in this model. Thus, we hypothesized that *Tie2* deficiency would exaggerate pulmonary vascular response to relevant environmental triggers of PAH by predisposing to EC apoptosis and microvascular degeneration.

Despite the evidence supporting a key role for the Ang1–Tie2 pathway in maintaining vascular homeostasis, it has previously been suggested that Ang1 may be causally related to the development of PAH (Thistlethwaite et al., 2001; Du et al., 2003). This was based on the observation that Ang1

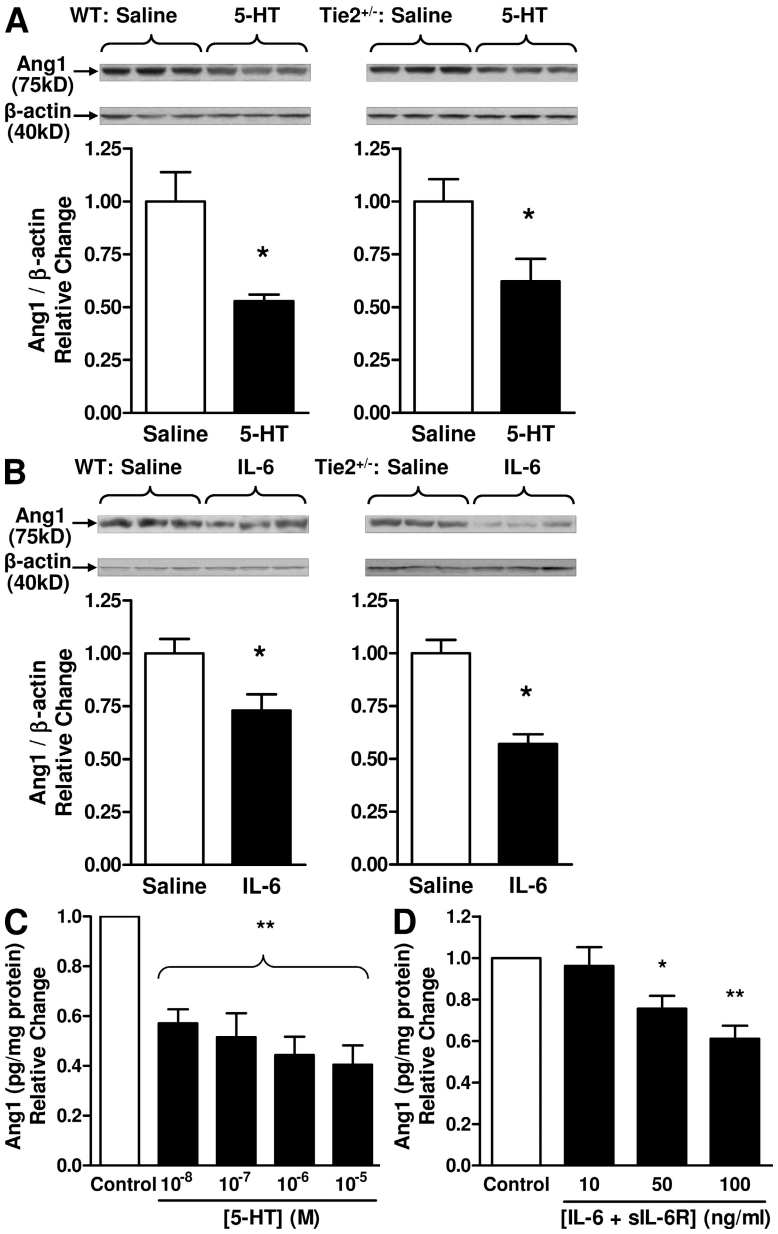

Figure 4. Effect of 5-HT or IL-6 on Ang1 protein levels in vivo and in vitro. (A) Decreased lung Ang1 protein levels in WT and *Tie2*$^{+/-}$ mice after exposure to 5-HT (A; $n = 5$ mice per group) or IL-6 (B; $n = 5$ mice per group) for 1 wk. (C and D) Decreased Ang1 protein secretion measured by ELISA in pulmonary artery SMCs serum starved overnight and stimulated with 10^{-8}–10^{-5} M 5-HT (C; $n = 4$ per group) or equal concentrations of a combination of IL-6 and sIL-6R (10, 50, and 100 ng/ml; D; $n = 4$ per group) for 24 h. Results in A and B are from two independent experiments; results in C and D are from four independent experiments. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$ versus saline (in vivo) or control (in vitro).

was robustly expressed in lung samples from associated PAH patients, and was strongly correlated with increases in pulmonary vascular resistance, whereas Ang1 protein and Tie2 activity were nearly undetectable in control human lung samples (Thistlethwaite et al., 2001; Du et al., 2003). However, these findings are in conflict with other reports showing that Ang1 is highly expressed in normal human and rodent lungs (Wong et al., 1997; Witzenbichler et al., 1998; Abdulmalek et al., 2001; Kugathasan et al., 2005), and an up-regulation of Ang1 in PAH has not been confirmed by other groups (Kugathasan et al., 2005; Dewachter et al., 2006). It is possible that the near-complete absence of Ang1 mRNA and protein expression in control lung samples in the initial Thistlethwaite report (Thistlethwaite et al., 2001) was related to their use of lobectomy samples from routine surgeries, which are often subjected to variability in processing speed and duration to storage before analysis. Indeed, we have found that the levels of Ang1 mRNA and protein fall off precipitously even after a 15-min delay in the processing of lung samples (unpublished data).

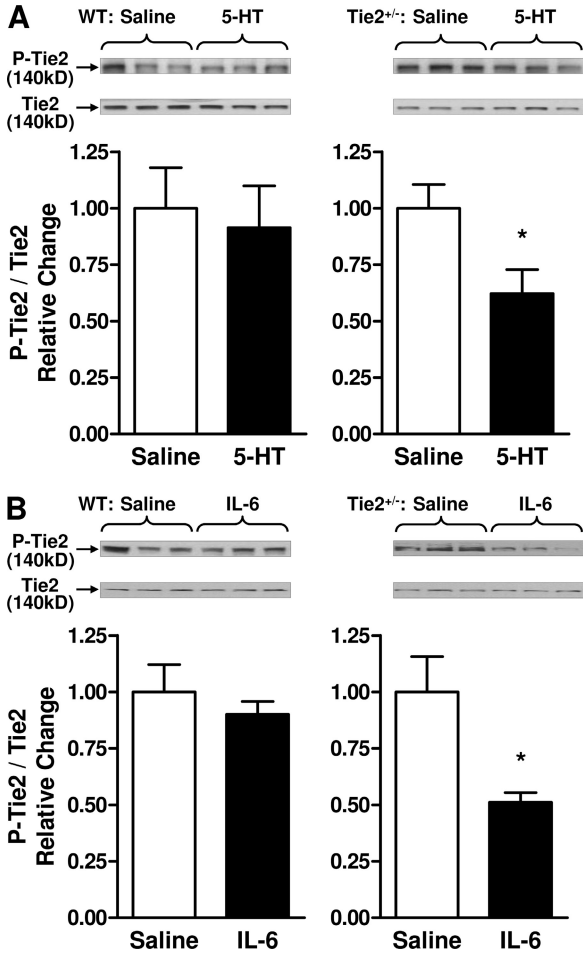

Figure 5. Effect of 5-HT or IL-6 on lung Tie2 activity. (A and B) Decreased activation of Tie2, assessed by normalizing levels of phosphorylated Tie2 to total Tie2, in *Tie2*$^{+/-}$ mice treated with 5-HT (A; $n = 4$ mice per group) or IL-6 (B; $n = 4$ mice per group) for 1 wk compared with saline-treated *Tie2*$^{+/-}$ mice. No change in Tie2 activation was observed in WT mice. Results in A and B are from two independent experiments. Data are presented as means ± SEM. *, $P < 0.05$ versus respective saline *Tie2*$^{+/-}$ mice.

The Thistlethwaite group also demonstrated that increasing Tie2 activity by *Ang1* gene transfer resulted in medial hyperplasia of pulmonary arterioles, vascular obstruction, and PAH (Sullivan et al., 2003; Chu et al., 2004), whereas inhibition of this signaling pathway via gene transfer of the extracellular domain of *Tie2* (soluble *Tie2*) was protective (Kido et al., 2005). However, patients with an activating mutation of *Tie2* show no evidence of PAH but rather develop large venous malformations, which exhibit either normal muscularization or vascular walls that are totally devoid of medial SMCs (Vikkula et al., 1996). Moreover, using a conditional transgenic *Ang1* overexpression strategy to avoid the confounding effects of viral vectors, we found no evidence of an increase in RVSP or RV remodeling even 9 wk after induction of *Ang1* gene expression. Furthermore, BT mice harboring both the tetracycline trans-activator and the *Ang1* transgenes showed no evidence of enhanced susceptibility to PAH in response to serotonergic stress, and if anything, this was blunted. These findings are consistent with previous studies in which nonviral *Ang1* gene transfer prevented PAH in rats in both the monocrotaline and hypoxia models (Zhao et al., 2003; Kugathasan et al., 2005).

The reasons for the marked discrepancies between our findings and those of the Thistlethwaite group are not clear; however, this may relate to differences in the experimental approaches. For example, their studies used viral vectors for gene transfer that can induce inflammation. It is also possible that differences in the overall magnitude of transgene expression (i.e., "dose") or spatial distribution may have contributed to these divergent results, and that higher levels of local Ang1 expression within the lung microvasculature could induce nonphysiological effects, such as increased muscularization and arterial remodeling. Finally, surgical manipulation such as the clamping of the pulmonary veins that was used to enhance the levels of transgene expression in the lung in these studies (Sullivan et al., 2003; Kido et al., 2005) could have damaged these delicate venous structures. It is well established in the electrophysiology literature that injury to pulmonary veins is an important cause of pulmonary hypertension (Robbins et al., 1998; Tsao and Chen, 2002; Yang et al., 2007). In our study, the use of a conditional, targeted transgenic model may have avoided some of these confounding effects.

Of note, we found an increase in basal levels of Ang1 in *Tie2*$^{+/-}$ compared with WT mice, which was associated with decreased Ang2 expression. This resulted in a marked increase in the ratio of the Tie2 agonist over the antagonist, which could serve to enhance activation of the remaining receptors, possibly in compensation for the effects of *Tie2* haploinsufficiency. Although overt PAH was not evident in most *Tie2*-deficient mice, abnormalities in distal microcirculatory perfusion were revealed by fluorescent microangiography, which were similar to those observed in a dominant-negative

BMPR2 transgenic mouse model (West et al., 2008) and consistent with subthreshold effects on vascular structure and function. In the present study, exposure to 5-HT or IL-6 alone did not induce a significant elevation in RVSP in WT mice; similarly, *Tie2* deficiency by itself resulted in only a minor effect. However, the combination of these genetic and environmental influences appeared to have "synergistic" effects resulting in the initiation of pulmonary apoptosis and the development of significant PAH. This is consistent with the "second-hit" hypothesis for the pathogenesis of this disease, in which the response to an environmental or endogenous trigger is enhanced by the presence of a sensitizing genetic abnormality (Newman et al., 2004; Song et al., 2005; Long et al., 2006; Song et al., 2008). In a manner analogous to the present findings, exposure to serotonergic (Long et al., 2006) or inflammatory stressors (Song et al., 2005) also produced an enhanced pulmonary hypertensive response in *BMPR2*$^{+/-}$ mice. Interestingly, as in *Tie2*-deficient mice, exposure to

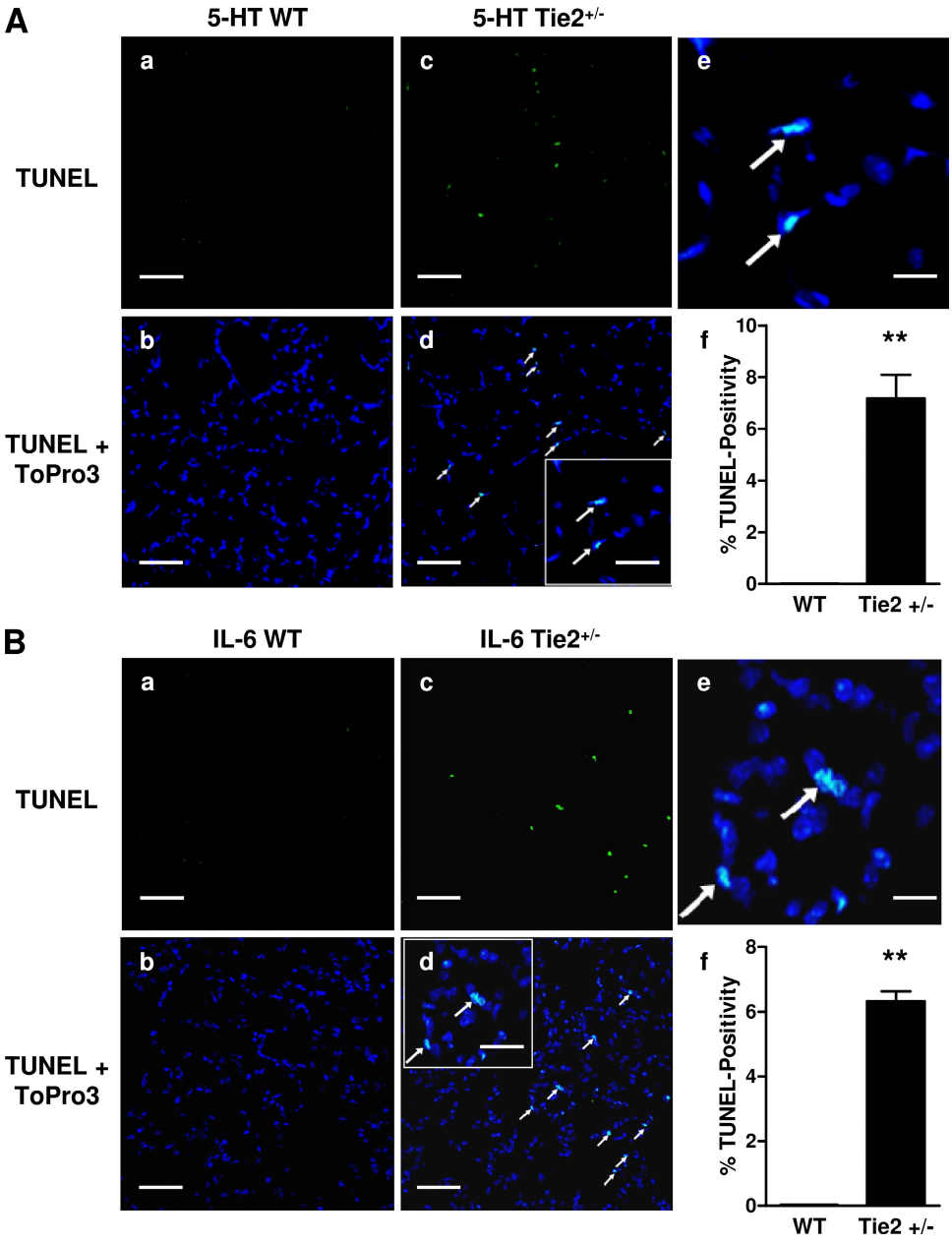

Figure 6. Effect of 5-HT or IL-6 on lung apoptosis. (A and B) Increased TUNEL staining in *Tie2*$^{+/-}$ compared with WT mice after exposure to 5-HT (A; $n = 4$ mice per group) or IL-6 (B; $n = 4$ mice per group) for 1 wk. TUNEL staining on 5-µm-thick paraffin-embedded lung sections from (a) WT and (c) *Tie2*$^{+/-}$ mice. Merged image of TUNEL and nuclear stainings (TO-PRO-3) of (b) WT and (d) *Tie2*$^{+/-}$ mice (arrows indicate TUNEL-positive cells). (e) Magnified view of TUNEL-positive cells (arrows) and (f) the percentage of TUNEL-positive cells. Results in A and B are from two independent experiments. Data are presented as means ± SEM. **, $P < 0.01$ versus WT. Bars: (a–d) 50 µm; (d, inset) 25 µm; (e) 12.5 µm.

chronic hypoxia did not result in greater PAH in *BMPR2*$^{+/-}$ compared with WT mice (Long et al., 2006).

Lung Ang1 protein levels were reduced by treatment with serotonergic or inflammatory mediators in both WT and *Tie2*$^{+/-}$ mice. This was associated with decreased Tie2 activation that was more pronounced in *Tie2*$^{+/-}$ compared with WT mice. Moreover, evidence of apoptosis was seen in the peripheral lungs in *Tie2*$^{+/-}$ mice exposed to 5-HT or IL-6 but not in WT littermates. Because Ang1 is primarily produced by periendothelial support cells, we examined the effect of these agents on Ang1 secretion by cultured pulmonary artery SMCs. In parallel to our findings in vivo, we observed a dose-dependent reduction in Ang1 secretion in response to both 5-HT and IL-6. Thus, in a *Tie2*-deficient genetic background, these mediators could act to interfere with an important compensatory mechanism and exacerbate the effect of haploinsufficiency on the activity of the Tie2 pathway.

5-HT and IL-6 may also exert direct effects on EC inflammation and survival. Both IL-6 and 5-HT are known to be elevated in the serum of patients with PAH (Hervé et al., 1995; Humbert et al., 1995; Itoh et al., 2006), and they have been shown to induce PAH in rats and mice (Eddahibi et al., 1997; Golembeski et al., 2005; Steiner et al., 2009). Increased circulating IL-6 has also been shown to be associated with increased evidence of EC apoptosis (Chirinos et al., 2005). As well, this mediator has been strongly implicated in EC activation and proinflammatory signaling in the context of PAH (Yoshio et al., 1997; Lesprit et al., 1998; Nishimaki et al., 1999). Recently, it has been suggested that increased susceptibility to PAH induced by *BMPR2* mutations may be attributable in part to the loss of inhibitory effects of BMPs on IL-6 (Hagen et al., 2007), resulting in a positive feedback loop of inflammatory cytokine signaling. Similarly, 5-HT has been strongly implicated in PAH, with idiopathic PAH patients demonstrating elevated circulating levels of 5-HT (Hervé et al., 1995). In experimental models, administration of dexfenfluramine (Dempsie et al., 2008), a 5-HTT substrate that also elevates extracellular levels of 5-HT (Rothman et al., 1999), has been shown to induce PAH in mice. In addition, several genetic and pharmacological models to either increase or decrease the activity of 5-HT receptors or the transporter have further highlighted the role of 5-HT in PAH (MacLean et al., 1996; Morecroft et al., 1999; Eddahibi et al., 2000; Keegan et al., 2001; Hironaka et al., 2003; Marcos et al., 2003; MacLean et al., 2004; Guignabert et al., 2005; Long et al., 2006; Morecroft et al., 2007), in part through its direct stimulatory effects on pulmonary arterial SMCs. However, the effects of 5-HT on EC growth and survival have not been as extensively studied. Lee et al. (1994) have reported that although 5-HT stimulated DNA synthesis in bovine pulmonary artery SMCs, this effect was not seen in pulmonary artery ECs. Similarly, we found no effects of 5-HT on EC growth and survival under basal conditions, but substantial EC apoptosis was demonstrated in response to 5-HT after siRNA-induced *Tie2* gene silencing. Thus, 5-HT may have differential effects on pulmonary vascular cells; although in SMCs it promotes proliferation, in ECs it may induce injury and apoptosis.

This is analogous to the effects of *BMPR2* deficiency or gene silencing, which also results in dysregulated SMC growth but unmasks increased EC apoptosis (Teichert-Kuliszewska et al., 2006). Moreover, we were able to establish that apoptosis was an important mechanism in the exaggerated PAH seen in *Tie2*-deficient animals in response to serotonergic stress, because this could be abrogated with the pan-caspase inhibitor Z-VAD. This is consistent with EC apoptosis playing a central role in the pathogenesis of PAH, possibly by

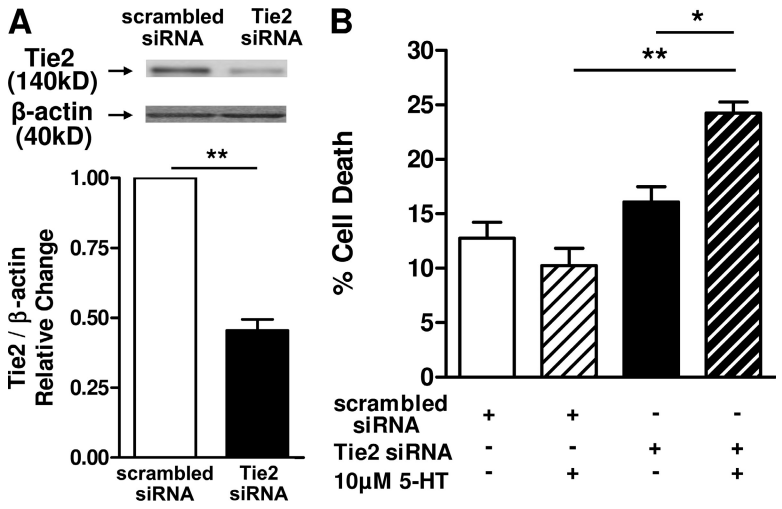

Figure 7. Effect of 5-HT on pulmonary artery ECs deficient in Tie2 expression. (A) Decreased Tie2 protein expression in human pulmonary artery ECs subjected to *Tie2*-specific silencing siRNA compared with control (scrambled siRNA; $n = 4$ per group). (B) Percentage of cell death by annexin V/PI–based flow cytometry in human pulmonary artery ECs treated with control (scrambled) or *Tie2* siRNA, and stimulated with 10 μM 5-HT for 24 h ($n = 4$ per group). Results in A and B are from four independent experiments. Data are presented as means ± SEM. *, $P < 0.05$; **, $P < 0.01$ as indicated.

leading directly to the degeneration and loss of function of distal precapillary arterioles. More importantly, these findings also emphasize the protective role of the Tie2 signaling pathway in preventing apoptosis and protecting against the development of PAH. Thus, interventions that inhibit the apoptotic cascade may be therapeutically useful in preventing the progression of PAH and need to be investigated.

In this study, we have shown that *Tie2* haploinsufficiency increased the susceptibility of transgenic mice to PAH, likely by sensitizing ECs to apoptosis in response to a second hit, such as exposure to 5-HT or a proinflammatory mediator.

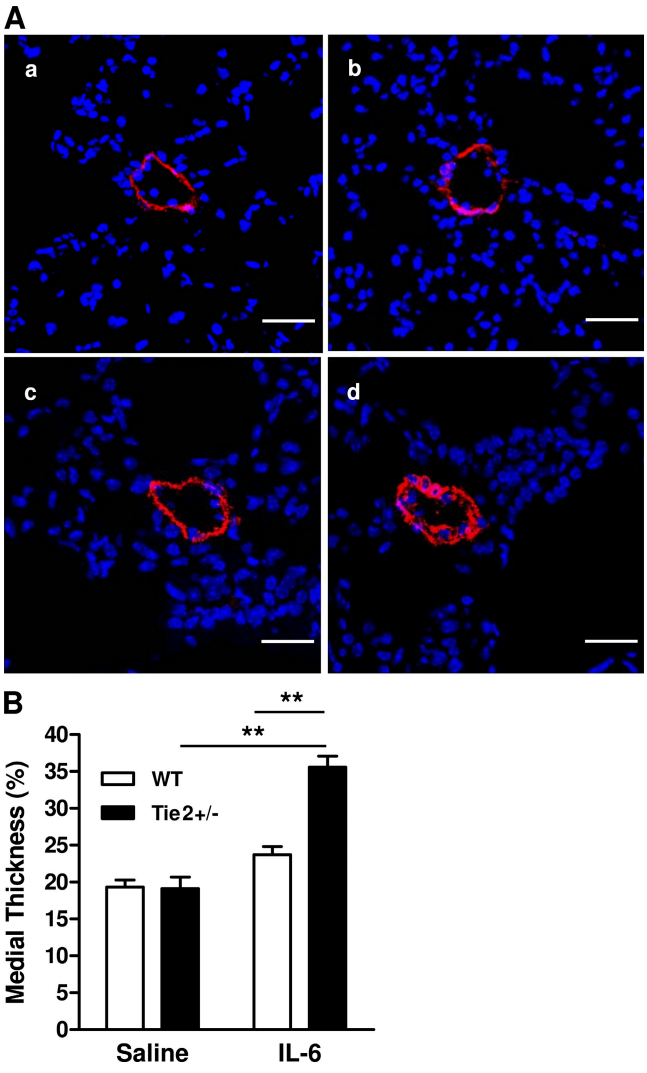

Figure 8. Effect of IL-6 on pulmonary arterial medial thickening. (A) Immunofluorescent images of vessels stained with α–smooth muscle actin from 2 wk of (a) saline-treated WT, (b) saline-treated *Tie2*$^{+/-}$, (c) IL-6–treated WT, and (d) IL-6–treated *Tie2*$^{+/-}$ mice (n = 4 mice per group). (B) Increased percentage of wall thickness of pulmonary arteries <50 µm in external diameter in *Tie2*$^{+/-}$ mice exposed to IL-6 for 2 wk compared with WT mice and compared with saline-treated *Tie2*$^{+/-}$ mice (n = 4 mice per group). Results are from two independent experiments. Data are presented as means ± SEM. **, P < 0.01 as indicated. Bars, 25 µm.

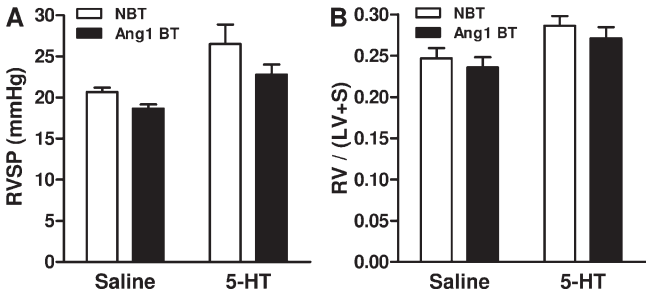

Figure 9. Effect of Ang1 overexpression on RVSP and RV hypertrophy. (A and B) No effect on RVSP (A; n = 6–10 mice per group) or RV hypertrophy (B; n = 6–10 mice per group) in NBT and *Ang1* BT mice treated with 5 nmol/h 5-HT for 1 wk. Mice were released from doxycycline suppression at 3 wk of age and exposed to 5-HT for 1 wk at 11–12 wk of age. Results are from three independent experiments. Data are presented as means ± SEM.

Both agents decreased Ang1 secretion, which in the *Tie2*-deficient background likely resulted in reduction in Tie2 activity below a critical threshold. In contrast to some previous reports, overexpression of Ang1 had no deleterious effects on pulmonary hemodynamics or vascular remodeling, a finding consistent with the now well-established role of Tie2 activity in maintaining vascular homeostasis. Collectively, these findings suggest that rather than promoting vascular remodeling and PAH, pulmonary Ang1 may be protective by supporting the basal activation of endothelial Tie2. Thus, these data may have important clinical implications by suggesting that therapeutic strategies based on inhibiting Tie2 activity may be harmful, whereas Ang1 and other Tie2 agonists may be useful in the treatment of this serious disorder.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Animal Care Committee of St. Michael's Hospital. Breeding pairs to generate *Tie2*$^{+/-}$ mice and *Ang1* BT mice were provided by D.J. Dumont (University of Toronto, Toronto,

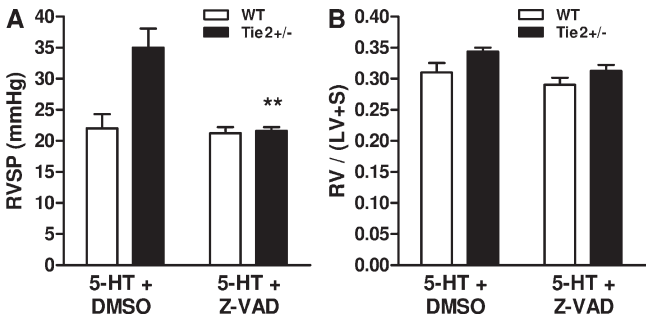

Figure 10. Effect of Z-VAD on 5-HT–treated WT and *Tie2*$^{+/-}$ mice. (A) Significant decrease in RVSP in 5-HT–treated *Tie2*$^{+/-}$ mice after simultaneous treatment with 3 mg/kg Z-VAD for 1 wk (n = 3–5 mice per group). (B) No significant change in RV hypertrophy was observed (n = 3–5 mice per group). Results are from two independent experiments. Data are presented as means ± SEM. **, P < 0.01 versus 5-HT + DMSO *Tie2*$^{+/-}$ mice.

Canada). WT littermates were used as controls in all experiments. *Tie2*$^{+/-}$ mice were generated by crossing *Tie2*$^{+/-}$ mice with WT mice. *Ang1* transgenic mice were generated by breeding Enh-*Tie2* tTA driver and pTET-*Ang1* responder mice. Ang1 overexpression was suppressed throughout breeding by administering mice with drinking water containing 100 µg/ml doxycycline (Sigma-Aldrich). After weaning at 3 wk of age, mice were released from doxycycline, allowing for Ang1 overexpression. NBT littermate mice were used as controls. Offspring genotypes were determined via PCR (see Genotyping).

Genotyping

Genomic DNA was extracted from mouse ear-notch samples, and PCR was performed using the buffers and PCR reaction mix from the REDExtract-N-Amp tissue PCR kit (Sigma-Aldrich), according to the manufacturer's instructions. A 0.5-µm mixture of the following primers was used in the PCR to detect the endogenous *Tie2* (~150 bp) and mutated *Tie2* (~450 bp): endogenous forward, 5′-AGCGCGTGGACCATGCGAGC-3′; endogenous reverse, 5′-CCATTGCTCAGCGGTGCTGTCCAT-3′; and mutant forward, 5′-AGGAGCAAGCTGACTCCACAG-3′. The PCR thermal cycling parameters included an initial denaturation at 94°C for 3 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 54°C for 40 s, and extension at 72°C for 30 s According to this, a final extension at 72°C for 10 min was performed, and the amplified DNA was resolved in a 1.5% agarose gel. *Ang1* BT mice were identified via PCR using the following primer pairs to detect the tetracycline transactivator (*tTA*; ~500 bp) and the human *Ang1* transgene (*hAng1*; ~350 bp): *tTA* forward, 5′-CTCACTTTTGCCCTTTAGAA-3′; *tTA* reverse, 5′-GCTGTACGCGGACCCACTTT-3′; *hAng1* forward, 5′-ATAGGAACCAGCCTCCTCTCT-3′; and *hAng1* reverse 5′-AAGGACACTGTTGTTGGTGGTA-3′.

Detecting plasma human Ang1 in *Ang1* BT mice

Cardiac puncture was performed on anesthetized mice, and blood was collected from both NBT and Ang1 BT mice. Plasma was isolated and stored at −80°C. Human Ang1 expression in mouse plasma was determined by ELISA according to the manufacturer's instructions (R&D Systems).

In vivo experiments

Hypoxia-induced PAH. Groups of three mice were placed in sealed Plexiglas, normobaric chambers (12 × 5 × 7 in) for 1 wk. The O_2 concentration in the chamber was maintained at 8–10% by controlling the inflow rates from tanks containing mixed air and nitrogen. The O_2 concentration in the chamber was measured daily using an O_2 analyzer (Engineered Systems and Designs). Control mice were maintained in room air.

5-HT–induced PAH. *Tie2*$^{+/-}$ and WT littermate mice as well as *Ang1* BT and NBT littermate mice (11–12 wk old) received 5 nmol/h of 5-HT (Sigma-Aldrich) or saline (control) via s.c. placed osmotic mini pumps (Alzet Osmotic Pumps) over a 1-wk duration. In separate experiments, mice receiving either saline or 5-HT also received daily i.p. injection of 3 mg/kg of the pan-caspase inhibitor Z-VAD-FMK (R&D Systems) or DMSO (control) for 1 wk.

IL-6–induced PAH. WT littermates and *Tie2*$^{+/-}$ mice received s.c. injections of 200 ng/kg/d of recombinant mouse IL-6 (Sigma-Aldrich) or saline (control) over a 1- or 2-wk duration.

Hemodynamic evaluation

Systemic SBP was determined by inserting a 1.4F Millar catheter (Millar Instruments, Inc.) into the left ventricle of anesthetized mice (200 mg/kg ketamine:10 mg/kg xylazine i.p.). Similarly, RVSP was determined in anesthetized mice by inserting the 1.4F Millar catheter into the RV. RV hypertrophy was assessed by evaluating the mass ratio of the RV to the left ventricle plus septum.

Fluorescent microangiography

This technique was adapted from Dutly et al. (2006). In brief, mice were anaesthetized and the pulmonary vessels were flushed through the RV with a flushing buffer (10 mM sodium phosphate, 127 mM sodium chloride, 10 mM EDTA, 5 U/ml heparin). A 10% mixture of 0.2 µm of fluorescent yellow-green microbeads (FluoSpheres; Invitrogen) reconstituted in low-temperature melting point agarose was injected into the pulmonary circulation via the RV. Crushed ice was then placed atop the mice to enhance gelling of the microbead–agarose mixture. After this, the lungs were inflated with cold 4% paraformaldehyde (PFA), and the heart–lung block was fixed in 4% PFA for 48 h and transferred to PBS for storage. The whole top right lobe was subjected to fluorescent microscopy (1× magnification; Discovery V8 stereo microscope equipped with an AxioCam MR3 camera) and images were acquired using AxioVision LE 4.6 software (all from Carl Zeiss, Inc.).

In vivo apoptosis analysis

This method was adapted from Cho et al. (1995). 5 µmol/kg PI (Sigma-Aldrich) was injected via the right external jugular vein of anesthetized mice (200 mg/kg ketamine:10 mg/kg xylazine) and allowed to circulate for 15 min before the mice were euthanized. The left atrium was transected, and the systemic and pulmonary circulations were flushed with PBS. Lungs were fixed in optimum cutting temperature and stored at −80°C overnight. The next day, 60-µm-thick cross sections of the left lobe of the lungs were cryosectioned for confocal microscopy. Cells that did not exclude the PI were considered to be nonviable.

TUNEL

Apoptotic cells were detected in 5-µm-thick paraffin-embedded sections using a fluorescent TUNEL assay kit (Promega) according to manufacturer's instructions. Nuclear counterstaining was performed using TO-PRO-3 (1:2,500; Invitrogen), and sections were examined by confocal microscopy (40× magnification; Radiance 2100) and images were acquired using LaserSharp software (both from Bio-Rad Laboratories). The number of TUNEL-positive nuclei was determined by examining four randomly selected microscopic fields from each of four to five mice from each experimental group. The percentage of TUNEL positivity was calculated as follows: (number of TUNEL-positive nuclei)/(total number of nuclei) × 100%.

Western immunoblotting

Protein was extracted from the lungs of WT and *Tie2*$^{+/-}$ mice and subjected to immunoblotting. Membranes were incubated overnight at 4°C with rabbit eNOS antibody (1:1,000; Cell Signaling Technology), rabbit Phospho-Tie2 antibody (1:1,000; Cell Signaling Technology), rabbit Tie2 antibody (1:1,000; Santa Cruz Biotechnology, Inc.), rabbit Ang1 antibody (1:1,000; Abcam), or goat Ang2 antibody (1:500; Santa Cruz Biotechnology, Inc.), and equivalent protein loading was demonstrated using mouse β-actin antibody (1:5,000; Sigma-Aldrich).

Muscularization analysis

5-µm-thick paraffin-embedded sections were stained with a Cy3-conjugated monoclonal α–smooth muscle actin antibody (1:200; Sigma-Aldrich), and nuclei were counterstained using TO-PRO-3 (1:2,500). Sections were examined by confocal microscopy (80× magnification; Radiance 2100) and images were acquired using LaserSharp software. The percentage of wall thickness was determined using the methodology by Beppu et al. (2004) and was as follows: % wall thickness = (WT1 + WT2)/(external diameter of vessel) × 100%, where WT1 and WT2 refer to wall thicknesses measured at two points diametrically opposite to each other. For each vessel, the percentage of wall thickness was calculated as the average determined from four regions of measurement.

Movat pentachrome staining

The Movat pentachrome staining was performed on 5-µm-thick paraffin-embedded sections to demonstrate extracellular matrix components such as fibrin, collagen, elastin, and proteoglycans/glycosaminoglycans. Vessels from four to five randomly selected microscopic fields (10× magnification) from each of three mice from each experimental group were analyzed using

a bright-field upright light microscope (model E800) equipped with a digital camera (DXM 1200), and images were acquired using Act-1 software (all from Nikon).

Human pulmonary artery SMC culture

Human pulmonary artery SMCs (Lonza, Ltd.) at 70–80% confluency were serum starved in basal medium with 0.1% BSA for 24 h and exposed to 5-HT (Sigma-Aldrich) at concentrations of 10^{-8}–10^{-5} M for 24 h. In a separate experiment, serum-starved cells were exposed to a combination of human IL-6 and human sIL-6R (IL-6 + sIL-6R; Sigma-Aldrich), each at concentrations of 10, 50, and 100 ng/ml for 24 h. At the end of the experiments, supernatants were collected for ELISA and cells were harvested for protein quantification.

Detecting membrane-bound IL-6R

Protein was harvested from human pulmonary artery SMCs and human endothelial progenitor cells were cultured for 7 d (positive control). Membranes were incubated overnight at 4°C with rabbit IL-6R antibody (1:500; Santa Cruz Biotechnology, Inc.).

ELISA

Human Ang1 levels from human pulmonary artery SMC supernatants were measured according to the manufacturer's instructions (R&D Systems).

Human pulmonary artery EC Tie2 siRNA

siRNA-mediated inhibition of *Tie2* gene expression (Santa Cruz Biotechnology, Inc.) in human pulmonary artery ECs (Lonza, Ltd.) was performed according to the manufacturer's instructions, and control cells were subjected to scrambled siRNA transfection. After siRNA treatment, cells were exposed to 10^{-5} M 5-HT in basal medium with 0.2% FBS for 24 h. At the end of this treatment, cells were labeled with annexin V and PI (Annexin-V-FLUOS staining kit; Roche) and analyzed by flow cytometry to determine the percentage of cell death.

Statistical analysis

All data are presented as means ± SEM. For in vivo experiments, means of two groups were compared using either an unpaired *t* test, or when appropriate, the nonparametric Mann-Whitney *U* test. Differences among multiple means were determined by analysis of variance (ANOVA), followed by the Student-Newman-Keuls post-hoc analysis, or the nonparametric Kruskal-Wallis test followed by the Dunn's post-hoc analysis. For in vitro experiments, repeated-measures ANOVA was used when appropriate, followed by the Dunnett's post-hoc analysis.

Online supplemental material

Fig. S1 demonstrates that hypoxia did not elicit more significant PAH in $Tie2^{+/-}$ compared with WT mice. Fig. S2 confirms that the membrane-bound IL-6R is not expressed in pulmonary arterial SMCs in culture. Fig. S3 demonstrates that lung eNOS protein levels are not significantly altered in WT or $Tie2^{+/-}$ mice after exposure to 5-HT, although a trend toward a decrease in eNOS protein levels was present in the $Tie2^{+/-}$ mice. Fig. S4 illustrates the presence of nonviable cells in the lungs of 5-HT–treated $Tie2^{+/-}$ mice via in vivo PI uptake. Fig. S5 demonstrates the absence of any overt phenotypic differences after Movat pentachrome staining between WT and $Tie2^{+/-}$ mice treated with 5-HT or IL-6. Fig. S6 confirms the expression of human Ang1 protein in the plasma of *Ang1* BT mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090389/DC1.

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