Endothelin B Receptor Deficiency Predisposes to Pulmonary Edema Formation via Increased Lung Vascular Endothelial Cell Growth Factor Expression

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Abstract—Endothelin (ET) may contribute to pulmonary edema formation, particularly under hypoxic conditions, and decreases in ET-B receptor expression can lead to reduced ET clearance. ET increases vascular endothelial cell growth factor (VEGF) production in vitro, and VEGF overexpression in the lung causes pulmonary edema in vivo. We hypothesized that pulmonary vascular ET-B receptor deficiency leads to increased lung ET, that excess ET increases lung VEGF levels, promoting pulmonary edema formation, and that hypoxia exaggerates these effects. We studied these hypotheses in ET-B receptor–deficient rats. In normoxia, homozygous ET-B–deficient animals had significantly more lung vascular leak than heterozygous or control animals. Hypoxia increased vascular leak regardless of genotype, and hypoxic ET-B–deficient animals leaked more than hypoxic control animals. ET-B–deficient animals had higher lung ET levels in both normoxia and hypoxia. Lung HIF-1α and VEGF content was greater in the ET-B–deficient animals in both normoxia and hypoxia, and both HIF-1α and VEGF levels were reduced by ET-A receptor antagonism. Both ET-A receptor blockade and VEGF antagonism reduced vascular leak in hypoxic ET-B–deficient animals. We conclude that ET-B receptor–deficient animals display an exaggerated lung vascular protein leak in normoxia, that hypoxia exacerbates that leak, and that this effect is in part attributable to an ET-mediated increase in lung VEGF content. (Circ Res. 2003;93:456-463.)

Key Words: hypoxia vascular permeability albumin extravasation HIF-1α

Pulmonary edema, characterized by leakage of intravascular proteins into the airspaces, occurs in many clinical conditions, including respiratory infections and acute respiratory distress syndrome (ARDS). The basic mechanisms leading to increased vascular permeability and pulmonary edema formation in these circumstances remain uncertain. Some evidence suggests that the vasoconstrictor peptide endothelin (ET) may contribute to this phenomenon. For example, patients with ARDS have elevated plasma levels of ET, and infusion of ET causes pulmonary edema formation in isolated perfused rat lungs. Also, ET receptor antagonists ameliorate experimental pulmonary vascular leak caused by oleic acid, leukotoxin, or exposure to hypoxia after viral infection. Exposure to hypoxia alone also leads to increased lung water in some species, including the development of high-altitude pulmonary edema (HAPE) in humans. The mechanisms by which exposure to hypoxia leads to pulmonary edema formation also remain uncertain, although at least in humans intense pulmonary vasoconstriction and elevated pulmonary artery pressures are thought to be critical factors. ET, a potent pulmonary vasoconstrictor, also may contribute to the pathogenesis of HAPE, given that plasma ET levels are elevated in humans with HAPE.

ET acts through two receptor subtypes, the ET-A and ET-B receptors. Activation of vascular ET-A receptors leads to vasoconstriction, whereas activation of vascular ET-B receptors leads to either nitric oxide–mediated vasodilation (when endothelial ET-B receptors are activated) or to vasoconstriction (when smooth muscle ET-B receptors are activated). The ET-B receptor is thought to internalize after ET binding, acting to reduce circulating levels of ET peptide. Alterations in pulmonary ET receptor expression could thus contribute to pulmonary edema formation, particularly decreases in ET-B receptor expression or activity. An animal model of ET-B receptor deficiency has been previously described, and the pulmonary vascular phenotype of these animals has been characterized. These transgenic animals do not express the ET-B receptor in the pulmonary vasculature, they have increased plasma ET content and pulmonary artery pressures even under normoxic conditions, and they develop exaggerated pulmonary hypertension when exposed to chronic hypoxia. Whether these animals are more susceptible to pulmonary edema formation has not been previously reported.
Another endogenous factor associated with pulmonary edema formation is the proangiogenic molecule, vascular endothelial growth factor (VEGF). VEGF is well-known to cause increases in vascular permeability, overexpression of VEGF in the lung leads to pulmonary edema formation in experimental animals, and VEGF has been implicated in acute lung injury in humans. In addition, several in vitro studies have demonstrated that, at least in some cell types, ET can act via the ET-A receptor to stimulate the production of VEGF mRNA and protein. This ET-mediated stimulation of VEGF production occurs via increases in the expression of the transcription factor hypoxia-inducible factor (HIF-1), even under normoxic conditions.

We hypothesized that deficiency of the ET-B receptor in the lung would lead to increased susceptibility to pulmonary vascular leak and edema accumulation, particularly in the presence of an additional stress such as hypoxia. We additionally hypothesized that these changes in pulmonary fluid balance would result from increased levels of ET in the lung acting at the ET-A receptor to increase local production of VEGF, which in turn acts to increase vascular permeability. We tested these hypotheses by studying ET-B receptor-deficient rats, measuring pulmonary vascular leak as Evans blue-labeled albumin extravasation under normoxic and hypoxic conditions. We also measured lung levels of ET, VEGF, VEGF receptor-2 (VEGFR-2), and HIF-1 protein in those animals and inhibited the vascular leak in the ET-B-deficient animals using either an ET-A receptor antagonist or VEGF antagonists.

**Materials and Methods**

**Animals**
A colony of the following three genotypes of rats was studied: transgenic control (+/+), heterozygous ET-B-deficient (sl/+), and homozygous ET-B-deficient (sl/sl). The colony was established and maintained as previously described. The genotype of each animal was confirmed with polymerase chain reaction of genomic DNA using standard techniques as previously described. All animals were allowed free access to food and water and were subjected to a similar day and night light cycle. Animals were housed at Denver altitude (1600 m) unless otherwise noted. This study was conducted in accordance with both local institutional guidelines and the National Institutes of Health’s Guide on the Humane Treatment of Experimental Animals.

**Exposure to Hypoxia**
Animals in hypoxic test groups were weighed and then exposed to hypobaric hypoxia in a ventilated chamber (P_o2=0.5 atmosphere) for 48 hours. After removal from the hypobaric chamber, hypoxia-exposed animals were maintained in normobaric hypoxia (P_o2=0.1) while undergoing vascular leak measurements as described below.

**Measurement of Vascular Protein Leak**
The leakage of protein from the vascular space into the lungs was assessed by measuring the accumulation of an intravascular tracer, Evans blue-labeled albumin, in the peripheral lung. After exposure to room air or to 48 hours of hypoxia, animals underwent measurement of Evans blue-labeled albumin extravasation as previously described.

**Lung Histology**
To determine if the changes in albumin extravasation observed in the hypoxic ET-B receptor-deficient animals correlated with histologic evidence of pulmonary edema formation, lung tissue from hypoxic sl/sl animals (n=3) and normoxic control (n=3) animals was fixed using a microwave technique reported to maximize preservation of pulmonary edema. Fixed tissue was paraffin-embedded, and cut sections were stained with H&E before light microscopic examination.

**Lung ET Content**
To determine if the increases in vascular leak seen in the normoxic and hypoxic ET-B-deficient animals were attributable to increased lung levels of ET, lung ET-1 peptide content was measured with a commercially available ELISA kit (BioMedica) using a previously published technique with minor modifications. Results were normalized to lung protein content.

**Western Blotting Studies**
To determine whether the increases in lung ET content were associated with changes in lung VEGF, VEGFR-2, or HIF-1 protein levels, lung homogenates were studied by standard Western blotting techniques. Membranes were reprobed for β-actin to confirm equal protein loading and transfer. The resulting images were scanned into a computer and analyzed by densitometry using NIH Image software. Results were expressed as arbitrary units, representing the ratio of VEGF, VEGFR-2, or HIF-1 to β-actin in each lane.

**ET Receptor Blockade**
To determine if activation of the ET-A receptor contributed to the vascular leak seen in the hypoxic ET-B receptor-deficient animals, a group of sl/sl animals (n=30) received sitaxsentan (15 mg/kg per day), an orally active ET-A receptor antagonist (generous gift of ICOS-Texas Biotechnology, L.P., Bellaire, Tex.), administered in their drinking water for 5 days before as well as during exposure to hypoxia for 48 hours. Animals were given the drug before hypoxic exposure to minimize dosing variability because of individual variations in water consumption with initial exposure to sitaxsentan and during hypoxic exposure. Exposure to hypoxia and measurement of Evans blue-labeled albumin extravasation was performed as described above.

**VEGF Antagonists**
To determine if VEGF contributed to the vascular leak seen in the hypoxic ET-B receptor-deficient animals, a group of sl/sl animals (n=4) received a single intraperitoneal injection (5 μg) of VEGF-neutralizing goat polyclonal antibody (R&D Systems, Minneapolis, Minn), a regimen previously reported to inhibit VEGF-induced angiogenesis, and then underwent exposure to hypoxia and vascular leak measurement as described above. To confirm the results of this experiment, an additional group of sl/sl animals (n=4) received a single subcutaneous injection (25 mg/kg) of VEGF-Trap, a soluble composite decoy receptor for VEGF (generous gift of Regeneron Pharmaceuticals, Tarrytown, NY) and then underwent exposure to hypoxia and vascular leak measurement as described above.

**Hemodynamic Studies**
To determine if the increase in labeled albumin extravasation seen in the hypoxic sl/sl animals was attributable to impaired left ventricular function, groups of normoxic control animals (n=3) and hypoxia-exposed sl/sl animals (n=3) underwent measurement of left ventricular end-diastolic pressure (LVEDP) by placement of a Millar catheter into the left ventricle via the carotid artery. To determine if the reduction in labeled albumin extravasation seen in hypoxia-exposed sl/sl animals receiving the VEGF-neutralizing antibody was attributable to a change in vascular permeability or to a change in pulmonary artery hemodynamics, pulmonary artery pressure was measured in hypoxia-exposed ET-B receptor-deficient animals with (n=4) and without (n=5) pretreatment with the VEGF-neutralizing antibody. Pulmonary artery pressure was measured by advancing a catheter from the right carotid artery into the pulmonary artery under the same anesthetic conditions.
Results

Greater Lung Vascular Leak in ET-B Receptor–Deficient Animals

To determine if ET-B receptor deficiency could lead to altered lung vascular leak, lung Evans blue–labeled albumin extravasation was measured under normoxic conditions in transgenic control animals (n=6), sl/+ ET-B receptor–deficient animals (n=5), and sl/sl ET-B receptor–deficient animals (n=4). The level of albumin extravasation was nearly identical in the control and heterozygous animals but was significantly increased compared with both other groups in the homozygous ET-B receptor–deficient animals (Figure 1, ANOVA P=0.02).

To determine if hypoxia would lead to an additional increase in lung vascular leak in these animals, lung Evans blue–labeled albumin extravasation was measured after exposure to hypoxia for 48 hours in transgenic control animals (n=6), sl/+ ET-B receptor–deficient animals (n=6), and sl/sl ET-B receptor–deficient animals (n=6). Animals exposed to hypoxia for 48 hours showed a significant increase in lung vascular leak compared with normoxic animals regardless of genetic background (Figure 1, P<0.04 for all three genotypes). Hypoxic sl/sl ET-B receptor–deficient animals had significantly greater albumin extravasation than hypoxic transgenic control animals (P=0.04), and hypoxic sl/sl ET-B receptor–deficient animals had an almost 4-fold increase in albumin extravasation compared with normoxic control animals. Hypoxic sl/+ animals were not significantly different from hypoxic sl/sl animals (P=0.42) or hypoxic control animals (P=0.12).

To determine if the changes in albumin extravasation observed in the hypoxic ET-B receptor–deficient animals correlated with histologic evidence of pulmonary edema formation, fixed lung tissue from hypoxic sl/sl animals and normoxic control animals was compared. Hypoxic sl/sl lungs were notable for extensive patches of thickened alveolar septa and smaller areas of proteinaceous debris in the alveolar spaces. No such changes were noted in the normoxic control lungs (Figure 2).

To determine if the increased albumin extravasation in the hypoxic sl/sl animals was attributable to reduced left ventricular function in those animals compared with normoxic control animals, measurements of LVEDP were obtained in those two groups. No difference in LVEDP was found (normoxic control, 3±1 mm Hg versus hypoxic sl/sl, 3±1 mm Hg).

ET-B Receptor Deficiency Leads to Increased Lung ET Content

To determine if the increases in vascular leak seen with ET-B receptor deficiency and exposure to hypoxia were associated with increases in lung ET-1 peptide content, lung tissue levels of ET-1 were measured in transgenic control animals and sl/sl animals under normoxic conditions and after exposure to hypoxia. As shown in Figure 3, under normoxic conditions, sl/sl animals (n=4) had elevated lung levels of ET-1 peptide compared with controls (n=5). Exposure to 48 hours of hypoxia led to a significant increase in lung ET-1 content in transgenic control animals (n=5). Lung ET-1 levels were greatest in the hypoxic sl/sl animals (n=7), which had significantly more lung ET-1 than did hypoxic control animals, although the difference from normoxic sl/sl animals did not reach statistical significance (P=0.09).

To determine if activation of the ET-A receptor contributed to the vascular leak seen in hypoxic ET-B receptor–deficient animals, sl/sl animals received the selective ET-A receptor antagonist sitaxsentan and then underwent measurement of Evans blue–labeled albumin extravasation after exposure to 48 hours of hypoxia. ET-A receptor blockade significantly reduced lung albumin extravasation under these circumstances from 251±55 to 91±25 ng/mg dry lung (P=0.02,
Figure 4), suggesting that much of the vascular leak in those animals was mediated by ET.

**ET-B Receptor Deficiency Leads to Increased Lung VEGF Expression**

To determine if the increases in vascular leak seen with ET-B receptor deficiency and exposure to hypoxia were associated with increased lung VEGF content, lung tissue levels of VEGF were measured by Western blotting in transgenic control animals and sl/sl animals under normoxic conditions and after exposure to hypoxia. ET-B receptor–deficient animals had significantly elevated levels of VEGF protein in the lung compared with controls under normoxic conditions (Figure 5; control, 1.9±0.3 versus sl/sl, 3.2±0.1 arbitrary units; *P*<0.01). Exposure to hypoxia did not significantly increase lung VEGF content in transgenic control animals (P=0.25), but exposure to hypoxia did increase lung VEGF content in sl/sl animals (Figure 5; normoxia, 3.2±0.1 versus hypoxia, 5.1±0.5 arbitrary units; *P*=0.03).

To determine if increases in vascular leak associated with ET-B receptor deficiency or hypoxia are attributable to changes in VEGF receptor expression, Western blotting studies were also done to assess VEGFR-2 protein levels in the lung. Neither ET-B receptor deficiency nor exposure to hypoxia led to a significant change in lung VEGFR-2 expression (ANOVA, *P*=0.28).

To determine if ET-A receptor activation contributed to the increased lung VEGF content found in the ET-B–deficient rats, lung VEGF content was measured by Western blotting in hypoxic sl/sl rats given sitaxsentan. ET-A receptor blockade significantly reduced lung VEGF content compared with untreated hypoxic sl/sl animals (*P*=0.02, Figure 6).

To determine if the increases in lung VEGF content were associated with ET-mediated increases in lung HIF-1α protein content, lung levels of HIF-1α were measured by Western blotting in normoxic control animals, hypoxic sl/sl animals, and hypoxic sl/sl animals given sitaxsentan. HIF-1α protein was virtually undetectable in the lungs of normoxic control animals but was clearly expressed in the hypoxic ET-B receptor–deficient animals (*P*<0.001, Figure 7). ET-A receptor blockade in the hypoxic sl/sl animals resulted in a significant reduction in HIF-1α protein (*P*<0.001), suggesting that ET contributed to the induction of HIF-1α protein in those animals.
VEGF Antagonism Reduces Lung Vascular Leak

To determine if the increased levels of VEGF protein in the hypoxic ET-B receptor–deficient animals contributed to the increased lung vascular leak seen in those animals, sl/sl animals were given a VEGF-neutralizing antibody before hypoxic exposure and then underwent measurement of Evans blue–labeled albumin extravasation after exposure to hypoxia for 48 hours. Pretreatment with neutralizing antibody to VEGF markedly reduced lung vascular leak (Figure 4, P<0.02 versus untreated animals). To confirm these results, an additional group of sl/sl animals received VEGF-Trap before hypoxic exposure and then underwent measurement of Evans blue–labeled albumin extravasation after exposure to hypoxia for 48 hours. Pretreatment with VEGF-Trap also markedly reduced lung vascular leak (Figure 4, P<0.02 versus untreated animals).

To determine if the effect of VEGF antagonism in reducing albumin extravasation was attributable to a change in lung vascular permeability or in pulmonary hemodynamics, pulmonary artery pressure was measured in sl/sl animals after exposure to hypoxia with or without pretreatment with VEGF-neutralizing antibody. Mean pulmonary artery pressure measured in room air was not significantly different in the animals receiving the VEGF-neutralizing antibody (P=0.03, Table). These findings suggest that the reduction in albumin extravasation in the antibody-treated animals was attributable to a reduction in vascular permeability rather than to altered pulmonary hemodynamics.

Discussion

In this study, we report our findings that rats lacking pulmonary vascular expression of the ET-B receptor demonstrate an increase in pulmonary vascular albumin extravasation under normoxic conditions and that subacute exposure to

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Animals were exposed to hypoxia for 48 hours and then underwent pulmonary artery pressure measurements breathing room air and 10% oxygen. Data are presented as mean±SEM, n=5 per group. *P=0.03 vs 10% oxygen/no antibody.
moderate hypoxia exaggerates that vascular leak. In addition, these animals also demonstrate an increase in lung VEGF content, which occurs because of activation of the ET-A receptor and is associated with increases in lung HIF-1α protein content. This increase in lung VEGF seems to increase vascular leak by increasing vascular permeability, because treatment with a VEGF-neutralizing antibody markedly reduces albumin extravasation in the lung without reducing vascular pressures. These findings provide the first in vivo evidence that alterations in lung ET content or ET receptor expression can play a role in promoting pulmonary edema formation by stimulating VEGF-mediated increases in pulmonary vascular permeability.

The central finding of this study was that animals with a deficiency of pulmonary vascular ET-B receptor expression demonstrated an increase in vascular protein extravasation into their lungs under both normoxic and hypoxic conditions. Histologic evidence of pulmonary edema found in the hypoxic ET-B–deficient animals suggests that the elevated albumin extravasation in those animals reflects early accumulation of both interstitial and alveolar edema. This association between ET-B receptor deficiency and vascular leak has not been previously reported, although ET-B receptor–deficient rats are known to have elevated circulating levels of protein content. This increase in lung VEGF in vitro, our results represent the first clear demonstration of this relationship in vivo. Interestingly, we not only found elevated VEGF levels in the lungs of ET-B–deficient rats under normoxic conditions but also that hypoxia led to an exaggerated increase in lung VEGF content in the ET-B receptor–deficient animals whereas it had little effect on control animals. Whereas most previously published work shows that chronic exposure to hypoxia increases VEGF expression in the lung, studies of the effects of acute hypoxic exposures have generated conflicting results, including three reports that acute hypoxia does not increase VEGF expression in the lung and two reports that it does. Our finding of markedly increased lung VEGF expression in the hypoxic ET-B receptor–deficient animals suggests, however, a synergistic effect of hypoxia and ET-B receptor deficiency on VEGF expression, perhaps as a result of ET-mediated stimulation of VEGF production. The fact that ET-A receptor blockade reduced lung VEGF content in the hypoxic ET-B receptor–deficient animals strongly supports this hypothesis. These results are consistent with previous in vitro work demonstrating in a variety of cell types that ET can act via the ET-A receptor to stimulate VEGF gene and protein expression. Our findings of increased HIF-1α protein content in the lungs of the hypoxic ET-B receptor–deficient animals and that ET-A receptor blockade reduces HIF-1α content suggest that ET can act via the ET-A receptor to increase lung HIF-1α levels. Although we did not prove that the ET-mediated increases in HIF-1α then contribute to the upregulation of lung VEGF, our results support that hypothesis. These results are also consistent with previous in vitro work showing not only that many factors increase HIF-1α expression via receptor-mediated interactions (as opposed to hypoxia-mediated stabilization) but also that ET-mediated increases in HIF-1α can lead to increased VEGF production.

VEGF is well known for its prominent vascular effects, promoting angiogenesis and increasing vascular permeability. We used two VEGF antagonists, neutralizing antibody and the VEGF-Trap soluble decoy receptor, to show that inhibiting VEGF markedly reduced pulmonary vascular protein extravasation in the hypoxic ET-B receptor–deficient rats. These results strongly suggest that increased expression of VEGF in the lung can contribute to vascular leak. These
results are also consistent with previous work showing that overexpression of VEGF in the lung leads to pulmonary edema formation and that high VEGF levels in the plasma of patients with ARDS increase permeability in cultured endothelial cells.13,14 The effects of VEGF on vascular permeability are believed to occur primarily via the VEGFR-2 receptor. Also in agreement with previous reports, we did not find an effect of either ET-B receptor deficiency or hypoxic exposure on VEGFR-2 expression,35 suggesting that changes in VEGF protein levels were the primary effectors of altered permeability in the animals studied. Finally, it is of note that even with VEGF antagonism, albumin leak in the hypoxic ET-B-deficient animals was still greater than in normoxic control animals, suggesting that factors other than VEGF may also contribute to hypoxia-induced changes in vascular permeability.

Our finding that the VEGF-neutralizing antibody did not reduce pulmonary artery pressure in the hypoxic ET-B receptor-deficient rats provides compelling evidence that the exaggerated albumin extravasation seen in those animals results from VEGF-induced increases in vascular permeability. The finding that VEGF blockade slightly increased pulmonary artery pressure is also consistent with previous reports that VEGF overexpression can protect against hypoxia-induced pulmonary hypertension.30 In addition, we found no difference in LVEDP between normoxic control animals and hypoxia-exposed ET-B receptor-deficient rats. Along with previous work showing that cardiac output in the sl/sl rat does not differ from transgenic control animals either in normoxia or after brief acute hypoxic exposure,13 these findings strongly suggest that altered cardiac function is unlikely to be a major factor in the increased albumin extravasation noted in the sl/sl animals or in the reduction in albumin extravasation noted with VEGF antagonism, although such an effect cannot be completely ruled out.

The possibility of additional alterations in gene expression in the ET-B-deficient animals deserves mention. We have previously shown by radioligand binding assays that lung ET-A receptor expression in the sl/sl animals is not markedly different from that in control animals.11 Chronic hypoxia, however, does lead to impaired pulmonary vascular nitric oxide and prostacyclin production in the sl/sl rats.12 Whether similar changes in endogenous vasodilator release occur with shorter-term hypoxic exposures and contribute to the increased vascular permeability we have observed remains to be studied.

In summary, then, our results suggest that a deficiency of lung ET-B receptor expression, especially when combined with exposure to moderate levels of hypoxia, results in increased lung ET peptide levels. Elevated levels of ET then act via ET-A receptor activation to increase lung VEGF content, perhaps mediated by increases in HIF-1α protein levels, and VEGF in turn acts to increase vascular permeability and promote pulmonary edema formation. These findings describe a chain of events that may be important in cardiorespiratory illnesses characterized by regional hypoxia and pulmonary edema formation and may ultimately suggest potential new sites of therapeutic intervention in the formation of pulmonary edema.

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References


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