

# Hyperoxia causes angiotensin 2–mediated acute lung injury and necrotic cell death

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The angiogenic growth factor angiotensin 2 (Ang2) destabilizes blood vessels, enhances vascular leak and induces vascular regression and endothelial cell apoptosis. We considered that Ang2 might be important in hyperoxic acute lung injury (ALI). Here we have characterized the responses in lungs induced by hyperoxia in wild-type and *Ang2*<sup>-/-</sup> mice or those given either recombinant Ang2 or short interfering RNA (siRNA) targeted to *Ang2*. During hyperoxia Ang2 expression is induced in lung epithelial cells, while hyperoxia-induced oxidant injury, cell death, inflammation, permeability alterations and mortality are ameliorated in *Ang2*<sup>-/-</sup> and siRNA-treated mice. Hyperoxia induces and activates the extrinsic and mitochondrial cell death pathways and activates initiator and effector caspases through Ang2-dependent pathways *in vivo*. Ang2 increases inflammation and cell death during hyperoxia *in vivo* and stimulates epithelial necrosis in hyperoxia *in vitro*. Ang2 in plasma and alveolar edema fluid is increased in adults with ALI and pulmonary edema. Tracheal Ang2 is also increased in neonates that develop bronchopulmonary dysplasia. Ang2 is thus a mediator of epithelial necrosis with an important role in hyperoxic ALI and pulmonary edema.

Supplemental oxygen is commonly administered to individuals with significant pulmonary or cardiac disease to increase the delivery of oxygen to peripheral tissues. Exposure to very high concentrations of oxygen ( $\geq 50\%$ ) for prolonged periods, however, causes hyperoxic acute lung injury (HALI). This response is characterized by endothelial and epithelial injury and enhanced alveolar capillary protein leak<sup>1–6</sup>. Studies from our laboratory and others have shown that HALI is caused by reactive oxygen species that mediate their effects, at least in part, by inducing a cell death response with features of necrosis<sup>2,4–7</sup>. Surprisingly, the mechanisms of hyperoxia-induced oxidant injury and cell death have not been adequately defined.

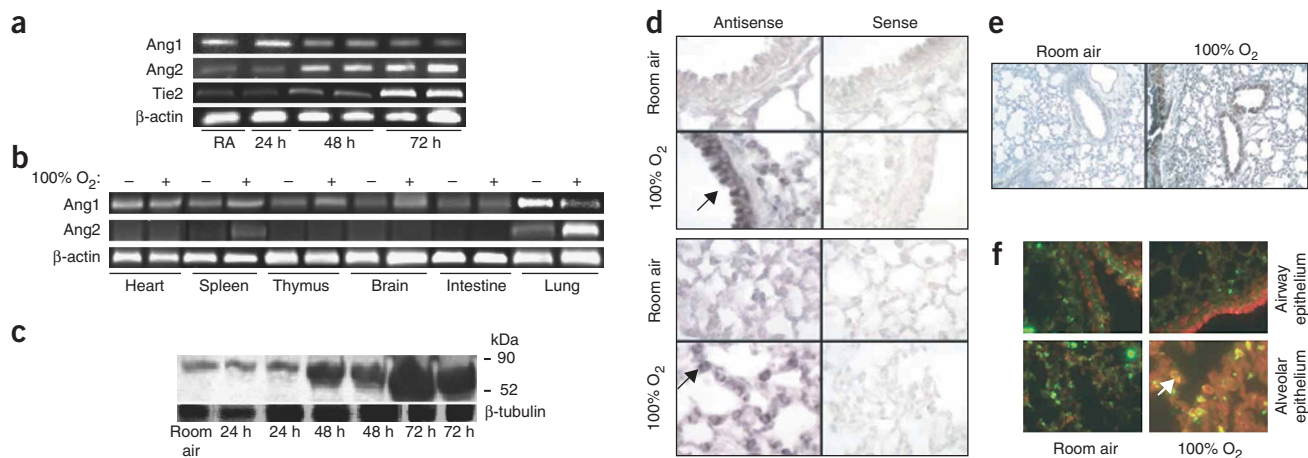
Angiogenesis and vascular remodeling are key events in fundamental physiological processes, including growth and development, wound healing, organ regeneration, inflammation and reproduction<sup>8–10</sup>. Each of these complex processes requires the coordinated production and interaction of multiple vascular regulating growth factors<sup>8,9,11–14</sup>. Vascular endothelial cell growth factor (VEGF) and angiotensin 1 (Ang1) have key roles in the angiogenic response: the former stimulates the generation of new, immature, leaky blood vessels, whereas the latter enhances angiogenesis, induces vascular maturation and decreases vascular permeability<sup>11–13</sup>. Angiotensin 2 (Ang2) is also

important owing to its ability to destabilize blood vessels, enhance vascular leak, antagonize Ang1 and, in the absence of other angiogenic stimuli, induce vascular regression and endothelial cell apoptosis<sup>15–17</sup>. Although Ang2 is stimulated in active wounds<sup>15,18</sup>, its role in the pathological tissue injury and edema observed in diseases such as HALI and other pulmonary edema states has not been characterized.

We considered that Ang2 might have a critical role in the pathogenesis of HALI. To test this hypothesis, we have characterized both the expression of angiotensins during HALI and the effects of hyperoxia in mice that produce Ang2 normally, in mice with null Ang2 mutations and in mice treated with siRNA targeted to Ang2. We have also defined the effects of recombinant Ang2 (rAng2) on mice in hyperoxia, and epithelial cells in normal and hyperoxic *in vitro* culture. These studies show that Ang2 is stimulated during the course of HALI and contributes to pathogenesis of the oxidant injury, DNA injury, cell death, inflammation, edema and mortality induced by hyperoxia *in vivo*. They also show that Ang2 induces epithelial cell necrosis in hyperoxia and that increased amounts of plasma, alveolar edema fluid (AEF), and tracheal aspirate Ang2 are present in human adults and neonates with ALI and pulmonary edema.

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**Figure 1** Effect of hyperoxia on Ang-1, Ang-2 and Tie 2. Mice were exposed to room air (RA) or 100% O<sub>2</sub> for up to 72 h. (**a–c**) Pulmonary Ang and Tie2 mRNA (**a**), pulmonary and extrapulmonary organ Ang mRNA (**b**) and BAL fluid Ang2 protein (**c**) were assessed by RT-PCR or western blotting. (**d**) *In situ* hybridization was used to localize Ang2 mRNA in the airways and parenchyma of mice exposed to room air or 100% O<sub>2</sub> for 72 h. Arrows highlight airway epithelial cells (top) and type II cells (bottom). (**e,f**) Immunohistochemical analysis was used to localize Ang2 protein in mice exposed to room air or 100% O<sub>2</sub> for 72 h. Single labeling experiments highlight prominent staining in airway and alveolar epithelial cells (**e**). Double labeling experiments highlight staining with anti-Ang2 (red) and anti-SP-C (green) antibodies (**f**). Arrow indicates a cell labeled with both antibodies. Original magnification,  $\times 40$  (**d–f**).

## RESULTS

### Effect of hyperoxia on Ang2 mRNA and protein

To address the role of Ang2 in HALI, we evaluated the levels of mRNA encoding Ang2 in mice at 24 h intervals after exposure to 100% O<sub>2</sub>. Ang2 mRNA was readily detected in lungs from wild-type mice breathing room air and increased markedly after exposure to 100% O<sub>2</sub> (**Fig. 1a**). This stimulation was seen after 48 h of exposure to 100% O<sub>2</sub>. It was simultaneously associated with an increase in expression of the Ang2 receptor Tie2 (**Fig. 1a**). These events were at least partially Ang2- and lung-specific because hyperoxia simultaneously decreased the level of Ang1 mRNA and an increase in Ang2 mRNA was not detected in other organs from mice exposed to 100% O<sub>2</sub> for up to 72 h (**Fig. 1b** and data not shown). This hyperoxia-induced alteration in Ang2 mRNA was associated with a concomitant increase in Ang2 protein in bronchoalveolar lavage (BAL) fluid from mice exposed to 100% O<sub>2</sub> as compared with room air (**Fig. 1c**).

We defined the sites of production and localization of Ang2 in lungs from mice breathing room air or 100% O<sub>2</sub>. *In situ* hybridization showed an increase in Ang2 mRNA in alveolar and airway epithelium, in alveolar type II cells and in some inflammatory cells after exposure to 100% O<sub>2</sub> (**Fig. 1d**). Similarly, immunohistochemical analysis showed an increase in Ang2 protein in airway epithelial cells and alveolar type II cells after exposure to 100% O<sub>2</sub> (**Fig. 1e,f**). These increases were Ang2-specific because *in situ* hybridization staining was not seen with sense probes, and immunohistochemical staining was not observed in the absence of primary antibody and was competed away by peptide excess (data not shown). Thus, hyperoxia is a potent inducer of Ang2 mRNA and protein in mouse pulmonary airway and alveolar epithelial cells.

### Role of Ang2 in HALI

To define the role of Ang2, we compared the effects of 100% O<sub>2</sub> in mice with wild-type and null mutant loci (*Ang2*<sup>-/-</sup>). In keeping with previous studies<sup>5,6,19</sup>, wild-type mice experienced ALI and death after 4–6 d of exposure to 100% O<sub>2</sub> (**Fig. 2a**). This response was associated with a significant increase in inflammatory cell accumulation, enhanced BAL macrophage and neutrophil recovery, a significant

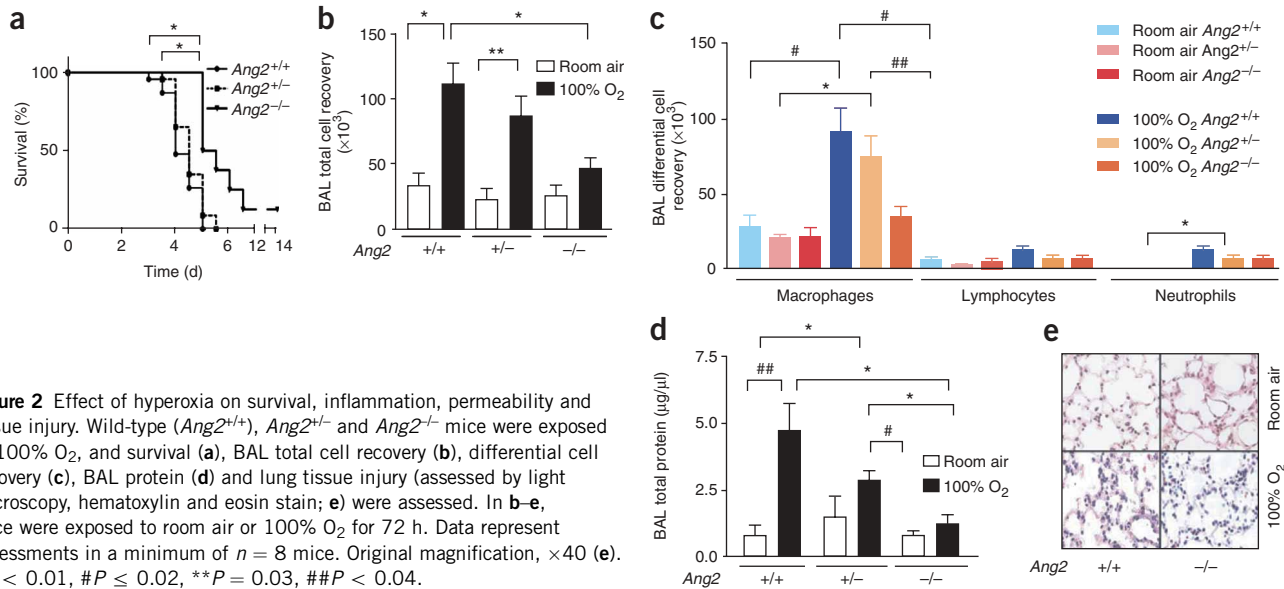
rise in alveolar capillary protein leak, and membrane blebbing with hyaline membrane accumulation (**Fig. 2b–e**). Ang2 was important in each of these responses because in its absence median survival in 100% O<sub>2</sub> was significantly enhanced (**Fig. 2a**), hyperoxia-induced inflammation (**Fig. 2b,c**) and alveolar capillary protein leak (**Fig. 2d**) were diminished, and membrane blebbing and hyaline membrane accumulation were ameliorated (**Fig. 2e**). These studies show that Ang2 has a critical role in pathogenesis of the mortality, inflammation and permeability alterations in HALI.

### Ang2 in oxidant injury and cell death

Because oxidant-induced DNA injury and cell death are important in the pathogenesis of HALI<sup>6,19</sup>, we evaluated the role of Ang2 in these responses. Exposure to 100% O<sub>2</sub> caused both oxidant injury that was readily apparent through tissue staining for 8-hydroxy-2'-deoxyguanosine (8-OHdG; **Fig. 3a**), and DNA injury and cell death that manifested as an increase in tissue staining for TdT-mediated dUTP nick end labeling (TUNEL; **Fig. 3b,c**). Ang2 had a critical role in the pathogenesis of both responses because oxidant-induced tissue injury 8-hydroxy-2'-deoxyguanosine (8-OHdG; staining) and TUNEL staining were significantly decreased in hyperoxia-challenged *Ang2*<sup>-/-</sup> mice as compared to wild-type mice (**Fig. 3a–c**). Thus, Ang2 is a critical mediator of hyperoxia-induced oxidant injury, DNA injury and cell death.

### Effects of rAng2 and Ang2 siRNA

*Ang2*<sup>-/-</sup> mice have abnormalities in their lymphatic system that could confound the interpretation of interventions that regulate the generation of tissue edema<sup>20</sup>. We therefore investigated whether the findings in *Ang2*<sup>-/-</sup> mice were the result of local lung effects of Ang2 or represented manifestations of the lymphatic abnormalities in these mice. To accomplish this, we examined the responses induced by hyperoxia both in mice that were given intraperitoneal rAng2 (or its vehicle control) and in mice that received intratracheal Ang2 siRNA (or an irrelevant control). rAng2-treated mice had increased hyperoxia-induced pulmonary vascular congestion, BAL and tissue inflammation and TUNEL-positive epithelial cell death as compared with vehicle-treated controls (**Fig. 4a–c**). Treatment with Ang2-specific



**Figure 2** Effect of hyperoxia on survival, inflammation, permeability and tissue injury. Wild-type ( $Ang2^{+/+}$ ),  $Ang2^{+/-}$  and  $Ang2^{-/-}$  mice were exposed to 100%  $O_2$ , and survival (a), BAL total cell recovery (b), differential cell recovery (c), BAL protein (d) and lung tissue injury (assessed by light microscopy, hematoxylin and eosin stain; e) were assessed. In b–e, mice were exposed to room air or 100%  $O_2$  for 72 h. Data represent assessments in a minimum of  $n = 8$  mice. Original magnification,  $\times 40$  (e). \* $P < 0.01$ , # $P \leq 0.02$ , \*\* $P = 0.03$ , ## $P < 0.04$ .

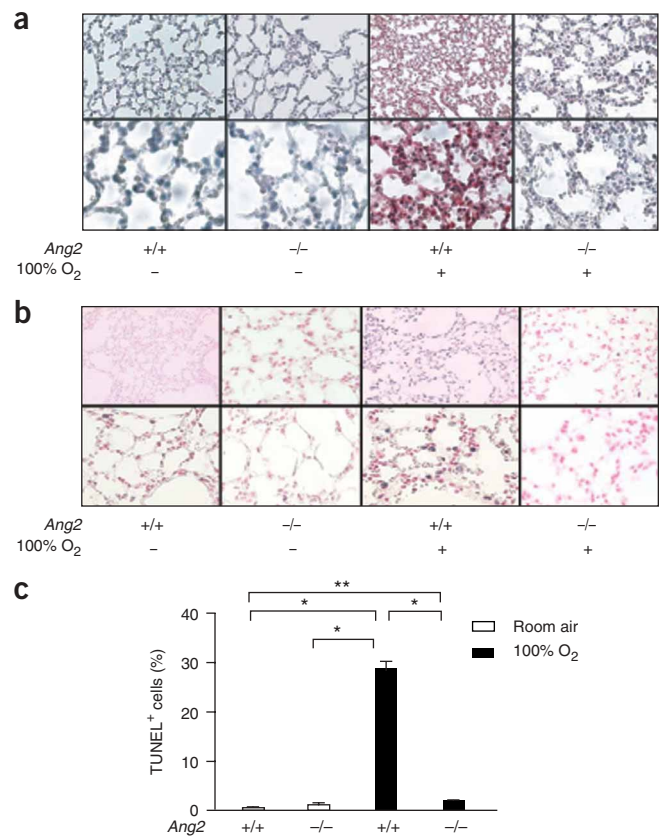
siRNA significantly decreased the levels of Ang2 mRNA. The level of mRNA encoding VEGF was not similarly inhibited (Fig. 4d), and the level of Ang2 mRNA was not altered by Bcl-2 siRNA (Supplementary Fig. 1 online). Ang2 mRNA inhibition was dose dependent: low-dose and high-dose Ang2 siRNA decreased hyperoxia-induced Ang2 mRNA accumulation to  $64.42 \pm 9.93\%$  and  $38.87 \pm 5.30\%$  of the respective scrambled siRNA controls ( $P \leq 0.01$ ; Fig. 4e). In addition, there was a proportionate decrease in Ang2 protein in the appropriate cellular compartment of the lung (Supplementary Fig. 2 online). In agreement with the results in  $Ang2^{-/-}$  mice and mice that received rAng2, Ang2 siRNA-mediated silencing significantly inhibited hyperoxia-induced inflammation and the TUNEL-positive cell death response (Fig. 4f,g). Taken together, these studies show that Ang2 is important in the pathogenesis of hyperoxia-induced tissue injury, cell death and inflammation.

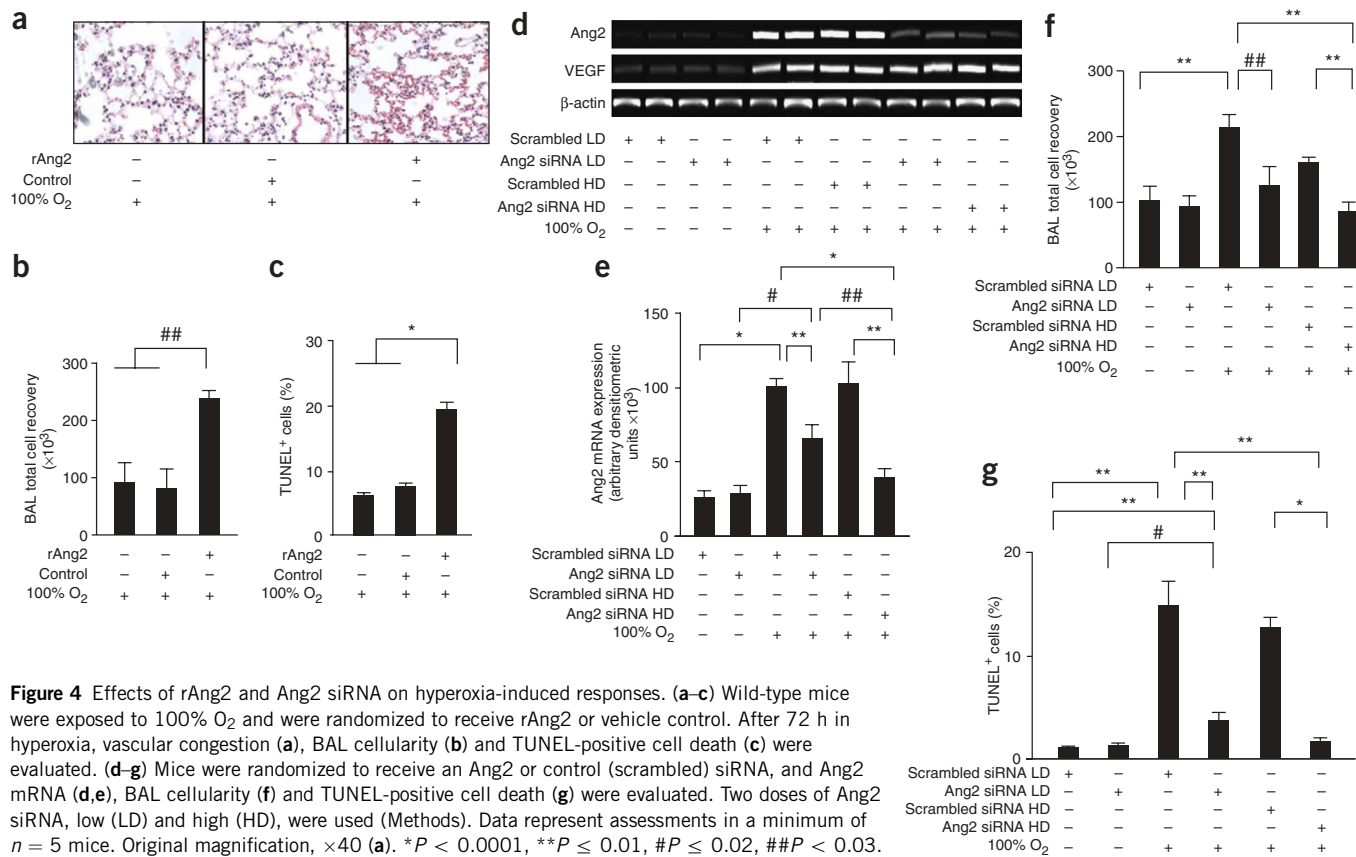
### Effects of Ang2 on caspases, cell death regulators, Ang4 and Tie2

We evaluated the role of Ang2 in regulation of the extrinsic and intrinsic cell death pathways. mRNAs encoding caspase-3, caspase-8, caspase-9, Fas and the BH3 domain-only proteins Bax, Bak, Bim and Bid were detected in lungs from wild-type mice breathing room air and increased significantly in mice exposed to 100%  $O_2$  for 72 h (Fig. 5a). In accordance with these observations, the bioactivity of caspase-3, caspase-8 and caspase-9 protein was also observed in lungs from mice breathing room air and increased significantly after hyperoxic challenge (Fig. 5b–d). The hyperoxia-induced increases in caspase mRNA and bioactivity, in addition to Fas, Bax, Bak, Bim and Bid mRNA, were reduced in  $Ang2^{-/-}$  mice subjected to hyperoxia (Fig. 5a–d). Thus, hyperoxia stimulates and activates the extrinsic and mitochondrial cell death pathways, critical initiator and effector caspases, and BH3 domain-only proteins through mechanisms that are, at least partially, dependent on Ang2.

**Figure 3** Role of Ang2 in hyperoxia-induced oxidant and DNA injury. (a,b) Wild-type and  $Ang2^{-/-}$  mice were exposed to room air (–) or 100%  $O_2$  (+) for 72 h, and subjected to both staining for 8-OHdG (a) and TUNEL evaluation (b). (c) Percentage of TUNEL-positive cells. Original magnification,  $\times 10$  (a, b, top);  $\times 40$  (a, b, bottom). \* $P < 0.0001$ , \*\* $P < 0.03$ .

We next examined the mechanisms by which hyperoxia regulates Tie2 expression and determined whether hyperoxia regulates other angiopoietins. Both Tie2 and Ang4 mRNAs were detected in lungs from mice breathing room air and were significantly increased in response to hyperoxia (Fig. 5a). The hyperoxia-induced increases in Tie2 and Ang4 were reduced in  $Ang2^{-/-}$  mice, and intermediate expression was seen in  $Ang2^{+/-}$  mice (Fig. 5a). Thus, hyperoxia





also stimulates the Ang2 receptor Tie2 and Ang4 through an Ang2-dependent mechanism.

### Ang2 induction of epithelial necrosis in hyperoxia

To define further the mechanisms of Ang2-mediated cell injury, we compared the effects of hyperoxia on MLE-12 mouse lung epithelial cells incubated in the presence and absence of supplemental Ang2. Cells cultured in 5% CO<sub>2</sub> and air did not show significant apoptosis or necrosis when incubated in the presence or absence of Ang2 (Fig. 5e,f). By contrast, 95% O<sub>2</sub> caused a modest increase in cellular necrosis, as measured by a selective increase in propidium iodide staining after 24–48 h in hyperoxia (Fig. 5e,f). This hyperoxia-induced necrotic response was significantly increased in the presence of supplemental Ang2 (Fig. 5e,f). This effect was dose dependent and most prominent after 48 h of incubation with Ang2 in hyperoxia. Thus, these studies show that Ang2 stimulates epithelial cell necrosis in hyperoxia *in vitro*.

### Ang2 in ALI and pulmonary edema

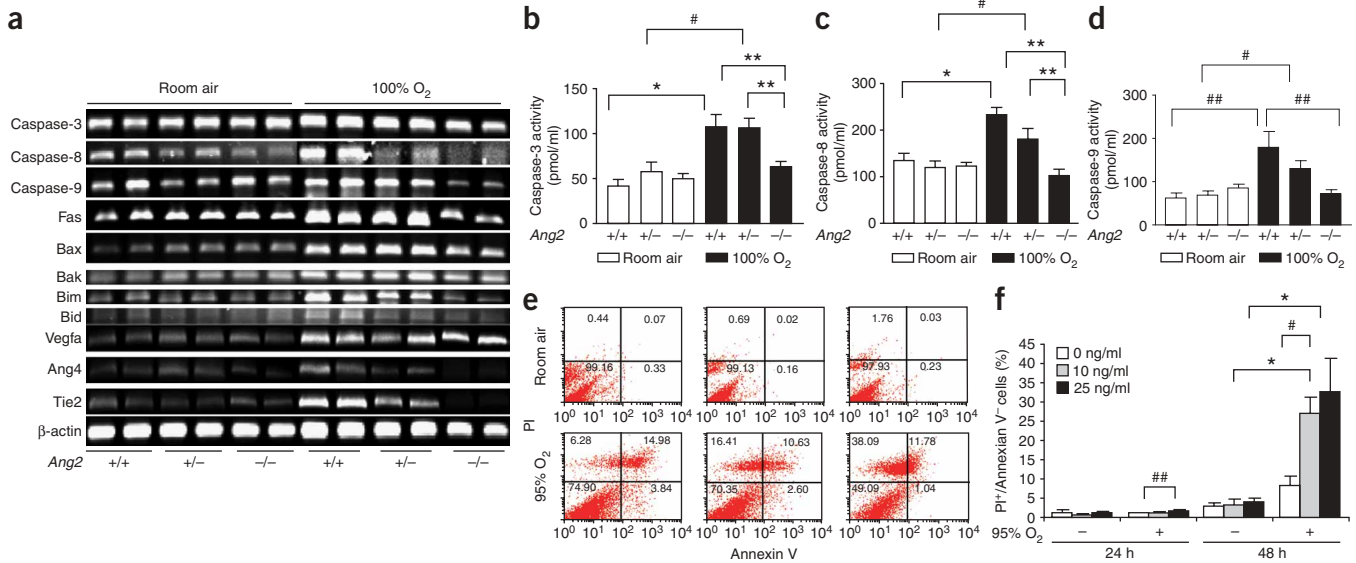
The studies noted above demonstrate that Ang2 is an important mediator of HALI that regulates oxidant-induced pulmonary edema. To evaluate the human disease relevance of these findings, we examined whether Ang2 is increased in the plasma or AEF of adults with ALI and/or hydrostatic pulmonary edema. The concentration of Ang2 was significantly higher in plasma from individuals with ALI than in plasma samples from controls and individuals with hydrostatic pulmonary edema ( $P \leq 0.05$ ). Similarly, higher concentrations of AEF Ang2 were seen in samples from individuals with ALI than in those with hydrostatic edema ( $P < 0.0001$ ; Fig. 6a). Ang2 was also apparent in tracheal aspirate from premature babies being ventilated for

respiratory distress syndrome (RDS). Some neonates with RDS develop HALI and pulmonary edema that subsequently lead to bronchopulmonary dysplasia (BPD) and even death. Of note, the concentration of tracheal aspirate Ang2 was significantly higher in babies that subsequently developed BPD and/or died ( $P < 0.01$ ; Fig. 6b). These studies show that Ang2 is increased in conditions characterized by exposure to high concentrations of oxygen and ALI in adult and neonatal individuals.

### DISCUSSION

Morphological studies in animal models have shown that toxic concentrations of oxygen initially induce focal endothelial cell cytoplasmic swelling and injury and, with continued exposure, necrosis of epithelial cells<sup>21–23</sup>. These alterations considerably increase alveolar capillary permeability and pulmonary edema. We considered that Ang2, a potent destabilizer of blood vessels, might contribute to the pathogenesis of HALI.

Our studies demonstrate that Ang2 and its receptor Tie2 are stimulated, whereas Ang1 is inhibited, by hyperoxic exposure. They also show that Ang2 both contributes to pathogenesis of the oxidant injury, DNA injury, cell death, inflammation, vascular leak and mortality seen after *in vivo* exposure to 100% O<sub>2</sub>, and augments hyperoxia-induced epithelial cell necrosis *in vitro*. In addition, Ang2 is increased in biological fluids from adult and neonatal individuals with ALI, indicating the relevance of these findings to human disease. Taken together, our studies show that Ang2 is a critical mediator of oxidant-induced lung injury and epithelial cell survival. They also suggest that the mechanisms that Ang2 uses to destabilize blood vessels during vascular remodeling may also be involved in the pathogenesis of injury and edema states in the lung or other tissues.



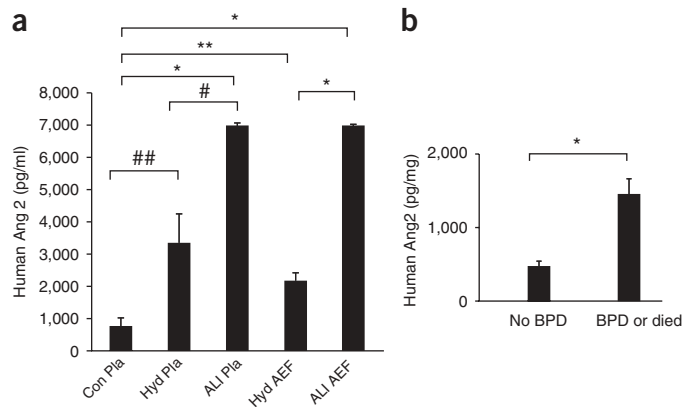
**Figure 5** Role of Ang2 in hyperoxia-induced alterations in apoptosis and angiogenic regulators and the effect of rAng2 on MLE-12 cell survival. (a–d) Wild-type, *Ang2*<sup>+/-</sup> and *Ang2*<sup>-/-</sup> mice were exposed to room air or 100% O<sub>2</sub> for 72 h, and the indicated mRNAs (a) and caspase-3, caspase-8 and caspase-9 bioactivity (b–d) were assessed. (e,f) MLE-12 cells were cultured for up to 48 h in 5% CO<sub>2</sub> and air, or 95% O<sub>2</sub> in the presence and absence of the indicated concentration of rAng2. (e) Apoptosis and necrosis were evaluated by Annexin V and propidium iodide (PI) staining and expressed as a percentage of the total cell number. (f) Percentage of cells undergoing pure necrosis (PI-positive, Annexin V-negative). \**P* < 0.01, #*P* ≤ 0.02, \*\**P* ≤ 0.03, ##*P* < 0.05.

Angiogenesis and vascular remodeling responses require Ang2-induced vascular destabilization to enable plasma proteins to leak from vessels to form a provisional vascular matrix<sup>15</sup>. In keeping with this concept, our studies show that Ang2 is induced in HALI. We found that in HALI Ang2 was produced by airway and alveolar epithelial cells and not by endothelial cells. This finding is not without precedent, however, because Ang2 production by transformed and non-transformed epithelial cells has been reported<sup>24</sup>. Because Ang2 blocks the effects of Ang1 (ref. 20), augments the vascular effects of VEGF<sup>25</sup>, and antagonizes Tie2 (refs. 8,15), it is easy to see how the Ang2 produced could contribute to the vascular destabilization and remodeling observed in HALI. Our demonstration that the vascular stabilizer Ang1 is simultaneously decreased would be expected to increase further the impact of these alterations. Ang2 does more than induce vascular destabilization, however, because it also contributed to local tissue oxidant injury, inflammation, edema and cell death in these studies.

The angiopoietin family of growth factors has four members, Ang1–Ang4, that bind to the tyrosine kinase receptor Tie2 (ref. 26). Our studies show that hyperoxia is an important modulator of this family that inhibits the expression of Ang1 and stimulates Ang2, Ang4 and Tie2. We found that VEGF, a powerful regulator of vascular growth and vascular permeability that interacts with angiopoietins, was also stimulated. Ang2 is a central mediator of many of these inductive responses because the hyperoxia-induced increases in Tie2 and Ang4 mRNA were mediated through an Ang2-dependent activation pathway. To our knowledge, this is the

first demonstration of an Ang2-dependent, angiopoietin effector cascade that regulates tissue injury, edema and inflammation. Ang1 and Ang4 are known to stabilize, whereas Ang2 and VEGF destabilize blood vessels<sup>27</sup>. Thus, the hyperoxia-induced pulmonary edema might be mediated, at least in part, by a combination of the decrease in Ang1, increase in Ang2 and/or the increase in VEGF. Alternatively, the induction of Ang4 could represent a feedback mechanism designed to control this important response.

Although cell death can be triggered by numerous stimuli and is mediated through an increasingly complex series of pathways, most cell death signals engage the cell death machinery at the level of the cell membrane or the mitochondria. The membrane (‘extrinsic’) pathway



**Figure 6** Ang2 in biological fluids from human adults and neonates. (a) Concentration of Ang2 in the plasma and undiluted AEF of individuals with ALI and healthy controls. Con, healthy adults; Hyd, adults with hydrostatic edema; ALI, adults with acute lung injury. *n* = 3–4 per group; \**P* < 0.0001, #*P* = 0.005, \*\**P* = 0.02, ##*P* = 0.05. (b) Concentration of Ang2 in the tracheal aspirate of premature babies affected with RDS with and without an adverse outcome (bronchopulmonary dysplasia and/or death). *n* = 5–9 per group; \**P* < 0.01.

triggers surface 'death receptors', such as Fas (which binds Fas ligand) and tumor-necrosis factor (TNF) receptor 1 (which binds TNF and lymphotoxin), which both activate caspase-8. Other stimuli use mitochondrial dysfunction to signal cell death. In this 'intrinsic' response, BH3 domain-only family members such as Bid are activated and interact with Bax-type proteins (Bax, Bak and Bok) to form or interact with mitochondrial pores, to release cytochrome *c*, to activate caspase-9 and to induce cell death<sup>19,28–30</sup>. To understand further the mechanism by which Ang2 contributes to the hyperoxia-induced cell death response, we characterized the cell death pathways activated in wild-type and *Ang2*<sup>-/-</sup> mice exposed to 100% O<sub>2</sub>. In accordance with previous reports<sup>5,6,19</sup>, these experiments showed that hyperoxia induces a brisk TUNEL-positive response with the activation of caspases and key components of both the extrinsic and intrinsic death pathways. Ang2 has a critical role in the pathogenesis of these responses because both TUNEL responses and activation of various caspases, Bax, Bak, Bim and Bid were decreased in the *Ang2*<sup>-/-</sup> mice. Hyperoxia-induced epithelial cell necrosis *in vitro* has been shown to be mediated by a Bid- and caspase-8-dependent pathway<sup>31</sup>. Our studies support this observation by demonstrating that Ang2 is a critical mediator of this necrotic response and regulator of these crucial cell death pathways *in vivo* and *in vitro*. Thus, Ang2 is a multifunctional regulator of oxidant-induced epithelial necrosis and has the ability to induce and/or to activate the extrinsic and intrinsic pathways, and initiator and effector caspases.

Hyperoxia causes a pulmonary endothelial and epithelial cell death response with features compatible with apoptosis including chromatin condensation and DNA fragmentation<sup>1,4–6,32</sup>; however, microscopy has shown that these lesions evolve into frank necrosis with continued hyperoxic exposure<sup>31</sup>. Our studies demonstrate that Ang2 is a critical contributor to the pathogenesis of HALI-induced cell death responses. In accordance with the context-dependent effector profile of Ang2 (refs. 8,15), Ang2 induced epithelial cell necrosis in hyperoxia but not under normal conditions. It is not clear whether these effects of Ang2 on epithelial cells are mediated directly through a receptor-mediated process or indirectly through other mechanisms. A receptor-mediated response cannot be ruled out, however, because Tie2 expression was induced by hyperoxia and Tie2 has been detected on normal and transformed epithelial cells<sup>33–35</sup>. In addition, we detected Tie2 mRNA in MLE-12 cells in our *in vitro* studies (data not shown). Coupled with studies showing that Ang2 blocks many of the effects of Ang<sup>36</sup>, induces endothelial cell apoptosis in the absence of VEGF<sup>15,37</sup>, and is produced at sites of cell death during cerebrovascular accidents and malignancies<sup>38,39</sup>, our results indicate that Ang2 is an important regulator of epithelial and endothelial cell survival in various sites and situations.

To assess the relevance of our findings to human disease, we measured Ang2 in plasma and undiluted AEF from adults with ALI, healthy controls and individuals with hydrostatic pulmonary edema<sup>40</sup>. Consistent with other studies<sup>16,38,39,41–44</sup>, Ang2 was detected in plasma from normal controls; however, plasma concentrations of Ang2 were markedly increased in individuals with ALI, and comparable amounts of Ang2 were found in AEF from these individuals. High concentrations of Ang2 were also observed in tracheal aspirate samples from neonates with RDS. It is not easy to interpret these values because healthy premature babies are not intubated and thus suitable control samples cannot be obtained. We found, however, that tracheal aspirate Ang2 was higher in newborns with RDS who had an adverse outcome (BPD and/or death) than in those that recovered quickly after surfactant administration. Because many of these individuals were on supplemental oxygen, we do not know whether the increase in Ang2 was due to the processes that initiated ALI or to the toxic

concentrations of oxygen that were contributing to and perpetuating their pathology. Nevertheless, these observations support the contention that Ang2 has a key role in the pathogenesis of these lung injury responses in adults and neonates, and suggest that interventions that block Ang2 production, receptor binding or signaling may be useful therapies in these settings.

Although significantly lower than in individuals with ALI, plasma and AEF Ang2 concentrations were increased in adults with hydrostatic pulmonary edema. Hemodynamic stressors and alterations in intravascular volume are classically thought to initiate hydrostatic pulmonary edema. As a result, the possibility that vascular-acting or other cytokines contribute to the pathogenesis of this response has not been addressed. Our studies might indicate that the hemodynamic and volume alterations that contribute to this disorder mediate their effects, in part, through induction of Ang2. In accordance with this, plasma interleukin-8, nitrites and nitrates are also increased in individuals with hydrostatic edema<sup>45,46</sup>.

In summary, our studies show that Ang2 is induced in HALI, where it has a critical role in pathogenesis of the oxidant injury, epithelial cell death and inflammation associated with this disorder. They also show that Ang2 is a potent stimulator of epithelial necrosis in hyperoxia and that Ang2 is increased in biological fluids from adults and neonates with ALI. In addition to ALI and pulmonary edema, an increase in Ang2, tissue edema and cell death is seen in other disorders, including cerebrovascular accidents, diabetic retinopathy and neurological neoplasms<sup>16,18,25,38,43,44,47–49</sup>. Our observations suggest that Ang2 is an important mediator of the injury, edema and/or cell death responses in these disorders and that Ang2 regulators may thus be therapeutically useful. Additional investigation of the effector profile of Ang2 in health, healing and disease and the consequences of Ang2 manipulation in these settings is warranted.

## METHODS

**Mice.** Transgenic *Ang2*<sup>-/-</sup> mice were generated and used as described<sup>20</sup>. The mice were generated from CBA × C57BL/6 zygotes and bred for more than ten generations onto a C57BL/6 genetic background. Unless otherwise indicated, wild-type and *Ang2*<sup>+/-</sup> littermates were used as controls. All mouse work was approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

**Oxygen exposure.** Mice aged 4–6 weeks were placed in cages in an airtight plexiglass chamber (55 × 40 × 50 cm<sup>3</sup>) and exposed to hyperoxia as described<sup>5,6,19</sup>.

**BAL.** Mice were killed, the trachea was isolated by blunt dissection and a small-caliber tube was inserted into the airway and secured. Two volumes of 1 ml of PBS containing 0.1% bovine serum albumin were instilled, gently aspirated, pooled and processed as described<sup>5,6,19</sup>.

**Histology.** Tissues were fixed overnight in 10% buffered formalin. After being washed in fresh PBS, the fixed tissues were dehydrated, cleared and embedded in paraffin by routine methods.

**Analysis of mRNA.** Mice were anesthetized, and the lungs were rapidly removed and frozen in liquid nitrogen. RNA was isolated from frozen lungs by using TRIzol Reagent (Life Technologies) in accordance with the manufacturer's instructions. RNA samples were treated with DNase and subjected to RT-PCR. The primers used in these assessments are given in **Supplementary Note** online.

***In situ* hybridization, immunohistochemistry, TUNEL and 8-OHdG evaluation.** These assessments are described in **Supplementary Note**.

**Antiserum generation and immunoblot analysis.** Immunoblot evaluation of Ang2 was done with a polyclonal rabbit antiserum to mouse Ang2

raised against a synthetic peptide corresponding to amino acids 264–283 (SPNSKSSVAIRKEEQTTFRD) of mouse Ang2 conjugated to keyhole limpet hemocyanin (Invitrogen). Lung lysate proteins were fractionated by SDS-PAGE, transferred to membranes and evaluated by western blotting. In these assays, the antiserum generated recognized the 70-kDa Ang2 isoform.

**siRNA generation and administration.** Duplex siRNAs were synthesized in a 2'-deprotected, desalted and purified form by Aplyam Pharmaceuticals. A potent Ang2 siRNA was identified by *in vitro* screening and selected for *in vivo* experimentation; siRNA characterization is described in **Supplementary Note** and **Supplementary Figures 1, 2, 4 and 5** online. Ang2 and irrelevant (scrambled control) siRNAs were delivered intranasally. The first dose was given just before the mice were subjected to hyperoxia; the second dose was given after 48 h of hyperoxia. Low (67 µg) and high (134 µg) doses of siRNA were used. Tissue samples were collected after 72 h of hyperoxia.

**Measurement of activity of caspases.** Lung homogenates were prepared and commercial kits were used to assess the activity of caspase-3 (Promega), caspase-8 and caspase-9 (Chemicon International) in accordance with the manufacturer's instructions.

**FACS analysis.** MLE-12 cells were grown to confluence, the indicated concentrations of Ang2 were added, and the cells were incubated for up to 48 h in 5% CO<sub>2</sub> and air or 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The medium and gases were refreshed daily. The cells were stained with Annexin V and propidium iodide using an Annexin V-FITC apoptosis detection kit (BD Biosciences), and analyzed by a flow cytometer (Becton Dickinson).

**Plasma, AEF and tracheal aspirate analysis.** Simultaneous plasma and undiluted AEF fluid samples were collected within 30 min of endotracheal intubation for severe acute respiratory failure from adults with ALI and from individuals with hydrostatic pulmonary edema as described<sup>40</sup>. Plasma was also obtained from normal healthy volunteers. Tracheal aspirate samples were collected from premature infants with RDS in the first 24 h of birth. BPD was defined as the need for oxygen with characteristic radiographic changes at 36 weeks postmenstrual age<sup>50</sup>. Tracheal aspirate samples were normalized to the amount of total protein. Samples were stored at -70 °C until assessment by an enzyme-linked immunosorbent assay (R&D Systems). Additional diagnostic information and human demographics are described in **Supplementary Note**.

All human work was approved by the Human Investigational Committee at Yale University School of Medicine and the Institutional Review Board at the University of California at San Francisco. Human work was done with informed consent.

**Statistical analyses.** Data are expressed as the mean ± s.e.m. As appropriate, groups were compared by the Student's two-tailed unpaired *t*-test or the log-rank test using GraphPad Prism 3.0 (GraphPad Software). A value of  $P \leq 0.05$  was considered statistically significant. Unless otherwise stated, representative figures reflect the findings in a minimum of  $n = 4$  evaluations and mean values reflect data obtained in a minimum of  $n = 5$  mice.

*Note: Supplementary information is available on the Nature Medicine website.*

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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