JNK2 up-regulates hypoxia-inducible factors and contributes to hypoxia-induced erythropoiesis and pulmonary hypertension

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The hypoxic response is a stress response triggered by low oxygen tension. Hypoxia-inducible factors (HIFs) play a prominent role in the pathobiology of hypoxia-associated conditions, including pulmonary hypertension (PH) and polycythemia. The c-Jun N-terminal protein kinase (JNK), a stress-activated protein kinase that consists of two ubiquitously expressed isoforms, JNK1 and JNK2, and a tissue-specific isoform, JNK3, has been shown to be activated by hypoxia. However, the physiological role of JNK1 and JNK2 in the hypoxic response remains elusive. Here, using genetic knockout cells and/or mice, we show that JNK2, but not JNK1, up-regulates the expression of HIF-1α and HIF-2α and contributes to hypoxia-induced PH and polycythemia. Knockout or silencing of JNK2, but not JNK1, prevented the accumulation of HIF-1α in hypoxia-treated cells. Loss of JNK2 resulted in a decrease in HIF-1α and HIF-2α mRNA levels under resting conditions and in response to hypoxia. Consequently, hypoxia-treated Jnk2−/− mice had reduced erythropoiesis and were less prone to polycythemia because of decreased expression of the HIF target gene erythropoietin (Epo). Jnk2−/− mice were also protected from hypoxia-induced PH, as indicated by lower right ventricular systolic pressure, a process that depends on HIF. Taken together, our results suggest that JNK2 is a positive regulator of HIFs and therefore may contribute to HIF-dependent pathologies.

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This article contains Fig. S1.

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2The abbreviations used are: PH, pulmonary hypertension; MEF, mouse embryonic fibroblast; COPD, chronic obstructive pulmonary disease; HIF, hypoxia-inducible factor; VHL, von Hippel–Lindau; EPO, erythropoietin; DMOG, dimethyloxalylglycine; DFO, deferoxamine; INOS, inducible nitric-oxide synthase; RBC, red blood cell(s); HPRT, hypoxanthine-guanine phosphoribosyltransferase; ANOVA, analysis of variance.
results in polycythemia and spleen enlargement due to splenic erythropoiesis (19).

JNK (also known as SAPK (stress-activated protein kinase)) is a member of the MAPK (mitogen-activated protein kinase) superfamily (20, 21). JNK is activated by a wide variety of extracellular stimuli, from pro-inflammatory cytokines to environmental stresses, including hypoxia (22). Upon activation, JNK regulates the activity of several transcription factors, including c-Jun, ATF-2, Elk-1, p53, and c-Myc, as well as other factors, such as members of the Bcl-2 family (23), and is involved in the regulation of many cellular activities from proliferation to cell death. Two of the three JNK isoforms, JNK1 and JNK2, are ubiquitously expressed among all tissues and are highly homologous to each other (24). They had been considered redundant isoforms; however, our group and others have previously demonstrated that JNK1 is the main JNK isoform that is activated by various extracellular stimuli, whereas the kinase activity of JNK2 is negligible (25). Rather, JNK2 targets its substrates for proteasomal degradation in unstimulated cells independent of its kinase function (26–30). The pathophysiological role of JNK1 and JNK2 under hypoxic conditions remains elusive. Here, we show that JNK2, but not JNK1, positively regulates HIFs in a murine model of ambient hypoxia and thereby contributes to hypoxia-induced PH and polycythemia.

### Results

**JNK2, but not JNK1, positively regulates HIF-1α in vitro**

It is known that both HIF-1α and HIF-2α proteins are constitutively degraded by the ubiquitin-proteasome system in oxygen-replete states but stabilized under conditions of low oxygen tension (31). To investigate whether JNK regulates HIF under resting and hypoxic conditions, we treated wild-type, Jnk1−/−, or Jnk2−/− mouse embryonic fibroblasts (MEFs) with normobaric hypoxia (1.5% oxygen, 5% carbon dioxide) for different times. In wild-type cells, HIF-1α proteins started to accumulate after hypoxia treatment for 15 min and peaked at 6 h (Fig. 1B). HIF-1α protein accumulation in hypoxia-treated Jnk1−/− cells was similar or slightly increased compared with that in wild-type cells (Fig. 1B). Silencing of JNK2 by siRNA in wild-type cells also resulted in decreased accumulation of HIF-1α in response to hypoxia (Fig. 1C), whereas silencing of JNK1 by siRNA in wild-
JNK2 up-regulates HIF contributing to hypoxia pathobiology

**JNK2 up-regulates HIF by maintaining HIF mRNA levels**

HIF-1α and HIF-2α proteins are constitutively degraded by the ubiquitin-proteasome system in oxygen-replete states, which is inhibited under hypoxic conditions, leading to their accumulation. We sought to determine whether our observation of decreased HIF-1α protein levels by loss of JNK2 under hypoxic conditions was due to increased HIF-1α proteasomal degradation. We treated wild-type and Jnk2−/− MEFs with the proteasome inhibitor MG132 under resting conditions and hypoxic conditions. In wild-type cells, MG132 treatment resulted in HIF-1α protein accumulation under resting conditions (Fig. 3A), consistent with the general notion that HIF-1α protein is subjected to constant proteasomal degradation in the steady state. Hypoxia treatment led to the accumulation of HIF-1α protein in wild-type cells, which was not further increased with MG132 (Fig. 3A). However, in Jnk2−/− cells, MG132 did not increase HIF-1α protein levels under either resting or hypoxic conditions (Fig. 3A), suggesting that the reduction in HIF-1α protein levels in Jnk2−/− cells is not due to accelerated proteasomal degradation. Interestingly, quantitative RT-PCR showed a drastic decrease in HIF-1α and HIF-2α mRNA expression levels in Jnk2−/− cells compared with wild-type cells under either resting or hypoxic conditions (Fig. 3, B and C). These data suggest that JNK2 is critical for maintaining HIF-1α and HIF-2α mRNA levels in vitro, which precedes hypoxia-induced accumulation of HIF proteins. To further investigate the role of JNK2 in maintaining HIF mRNA levels, we sought to determine whether JNK2 regulates the stability of HIF mRNAs. We treated wild-type or Jnk2−/− cells with actinomycin D, which blocks de novo mRNA synthesis (34). Using an equal amount of total RNA for cDNA preparation, we used the standard curve of dilutions to estimate mRNA copy number as described previously (35). As expected, HIF-1α and HIF-2α mRNA levels were lower in Jnk2−/− cells compared with wild-type cells at baseline (Fig. 3, D and E). After normalizing to the abundance of HIF-1α transcript at baseline, we observed a slower rate of degradation of HIF-1α transcript in Jnk2−/− cells compared with wild-type cells (Fig. 3F, slope of −1.054%/h in Jnk2−/− versus −6.309%/h in WT; p < 0.05). Similar results were obtained for HIF-2α transcript (Fig. 3G, −4.653%/h versus 0.7267%/h; not statistically significant). These results suggest that JNK2 up-regulation of HIF mRNAs is not mediated through stabilizing HIF transcripts.

**JNK2 up-regulates the expression of HIF under “pseudohypoxic” conditions**

We then determined whether JNK2 also up-regulates HIF in vitro under pseudohypoxic conditions without the use of ambient low oxygen tension. We treated wild-type or Jnk2−/− cells with either the prolyl-4-hydroxylase inhibitor dimethyloxalylglycine (DMOG) or the iron chelator deferoxamine (DFO), both of which stabilize HIF protein, but by different mechanisms; DMOG inhibits the prolyl hydroxylases that suppress HIF expression through VHL-dependent ubiquitination and proteasomal degradation, whereas DFO is an iron chelator that...

during hypoxia and that this regulation is independent of the kinase activity of JNK2.

**JNK2, independently of its kinase function, is both necessary and sufficient for HIF-1α expression**

To further investigate the role of JNK2 in HIF-1α up-regulation, we transfected Jnk2−/− cells with either empty vector or expression vector encoding HA-JNK2 and then treated the cells with hypoxia. Complementation of JNK2 into Jnk2−/− cells restored HIF-1α levels under hypoxic conditions (Fig. 2A). In addition, overexpression of JNK2 in wild-type cells further increased HIF-1α protein levels under hypoxic conditions (Fig. 2B). Reports by our group and others have shown that JNK2 can regulate gene/protein expression in a kinase-independent manner (33). To elucidate whether the kinase activity of JNK2 is required for its regulation of HIF-1α, we transfected Jnk2−/− cells with plasmids expressing wild-type JNK2 or either of the two kinase–deficient mutants of JNK2 (JNK2-KM, in which the catalytically active lysine 149 was mutated to methionine, or JNK2-APF, in which the consensus phosphorylation motifs threonine 183 and tyrosine 185 that are required for JNK2 kinase activation were replaced by non-phosphorylatable residues alanine and phenylalanine, respectively). Under hypoxic conditions, we observed similar levels of HIF-1α protein compared with wild-type JNK2–expressing cells (Fig. 2C). Taken together, these data provide evidence that JNK2 is both necessary and sufficient for the expression of HIF-1α protein...
JNK2 up-regulates HIF contributing to hypoxia pathobiology

A

| MEF | WT | Jnk2<sup>−/−</sup> |
|-----|----|-------------------|
| MG132 | - | + |
| Hypoxia | 0h | 0h |
| MW (kDa) | - | + |
| HIF-1α | 150 | 100 |
| β-actin | 37 |

B

HIF-1α mRNA Expression Level (normalized to HPRT)

C

HIF-2α mRNA Expression Level (normalized to HPRT)

D

HIF-1α mRNA Level (Relative Copy Numbers)

E

HIF-2α mRNA Level (Relative Copy Numbers)

F

HIF-1α degradation rate (percentage)

G

HIF-2α degradation rate (percentage)
JNK2 up-regulates HIF contributing to hypoxia pathobiology

displaces porphyrin iron and interferes with the heme oxygen sensor (36–40). Similar to the response to hypoxia, DMOG or DFO treatment increased HIF-1α protein levels in wild-type cells, which was again inhibited in Jnk2−/− cells (Fig. 4, A and B). Under these conditions, HIF-1α and HIF-2α mRNA levels were consistently lower in Jnk2−/− cells than in wild-type cells (DMOG (Fig. 4, C and D) and DFO (Fig. 4, E and F)). These data suggest that the regulation of HIF by JNK2 is not restricted to the stimulus of low oxygen tension, but is also observed in the context of alternative HIF-stabilizing mechanisms, including hydroxylase inhibition and iron chelation. To determine whether the regulation of HIF-1α and HIF-2α mRNA by JNK2 depends on the transcriptional activity of HIF per se, we treated wild-type and Jnk2−/− MEFs with the HIF-1α DNA-binding inhibitor, echinomycin, followed by hypoxia treatment. We first measured the mRNA levels of several well-established HIF target genes (Vegf, inducible nitric-oxide synthase (iNos), and glucose transporter 1 (Glut1)). Consistent with reduced HIF levels in Jnk2−/− cells, hypoxia-induced mRNA levels of Vegf, iNos, and Glut1 were decreased in Jnk2−/− cells compared with their wild-type counterparts (Fig. 4, G–I). As expected, echinomycin treatment dramatically inhibited the induction of Vegf, iNos, and Glut1 in hypoxic wild-type cells and further inhibited their induction in Jnk2−/− cells (Fig. 4, G–I). Under these conditions, the levels of HIF-1α or HIF-2α mRNA remained lower during hypoxia in Jnk2−/− cells compared with wild-type cells in the presence of echinomycin (Fig. 4, J and K). These data suggest that the JNK2 regulation of HIF mRNA occurs independently of HIF transcriptional activity.

Jnk2−/− mice have decreased erythropoiesis under hypoxic conditions

To determine whether the regulation of HIF by JNK2 occurs in vivo, we treated wild-type and Jnk2−/− mice (Fig. 5A) with hypoxia (7% oxygen) for 7 days. We analyzed the mRNA levels of HIFs in the kidney, where the HIF target gene Epo is produced (41). We observed decreased mRNA levels of HIF-1α and HIF-2α in Jnk2−/− mice as compared with wild-type mice under hypoxic conditions (Fig. 5, B and C), consistent with our in vitro results (Figs. 1–3). Accordingly, the protein levels of HIF-1α and HIF-2α in the kidney were reduced in hypoxic Jnk2−/− mice compared with wild-type mice under the same conditions (Fig. 5, D and E). The HIF protein levels in the brain were also decreased in hypoxic Jnk2−/− mice compared with hypoxic wild-type mice (Fig. 5F). We assessed the levels of EPO in the serum, and as expected, EPO was induced in wild-type mice under hypoxic conditions, which was suppressed in Jnk2−/− mice (Fig. 5G). These data suggest that JNK2 also maintains HIF expression in mice under hypoxic conditions.

Under hypoxic conditions, up-regulation of HIF expression, predominantly HIF-2, induces the transcription of their target gene EPO, leading to increased erythropoiesis (19, 42–45). To determine the in vivo relevance of JNK2 regulation of HIFs, we treated wild-type and Jnk2−/− mice with hypoxia (7% oxygen) for 7 days and examined hypoxia-induced erythropoiesis. Hypoxia treatment resulted in a drastic increase in erythropoiesis in wild-type mice, as shown by increased red blood cell (RBC) counts (Fig. 6A) and reticulocytes (Fig. 6, B and C) in the peripheral blood. However, in hypoxic Jnk2−/− mice, the total numbers of RBC and reticulocytes in the peripheral blood were less than those from wild-type mice under the same conditions (Fig. 6, A–C). It has been reported that hypoxia treatment leads to splenomegaly in mice due to increased erythropoiesis in the spleen (46). Consistent with this, we observed that wild-type mice had increased spleen/body weight ratio after hypoxia treatment for 7 days (Fig. 6, D and E). As expected, splenomegaly was alleviated in Jnk2−/− mice under the same conditions (Fig. 6, D and E).

To further confirm that Jnk2−/− mice have decreased medullary and extramedullary erythropoiesis, we performed flow cytometry to trace erythroid precursors in the spleen and bone marrow using the markers of CD71 (transferrin receptor) and Ter119 (glycoporphin A–associated protein). Ter119 is expressed at intermediate levels at the earliest, proerythroblast stage and is subsequently expressed at high levels during erythroid maturation (47). Meanwhile, CD71 expression declines with erythroid maturation (48). Using a gating strategy previously reported by others (19, 49, 50), we applied quadrants to define four subpopulations of erythroid cells in the spleen: Ter119lowCD71high (quadrant I, proerythroblasts), Ter119highCD71high (quadrant II, early erythroblasts), Ter119highCD71low (quadrant III, late erythroblasts), and Ter119lowCD71low (quadrant IV, non-erythroblasts) (Fig. 7A).

There were no differences in the numbers of total erythroid cells or different subpopulations of erythroid cells between wild-type and Jnk2−/− mice in the steady state (Fig. 7, A and B). Hypoxia treatment induced de novo erythropoiesis in wild-type mice, as shown by increased numbers in total erythroid cells as well as in the subpopulations of proerythroblasts and early erythroblasts (Fig. 7, A and B). In hypoxic Jnk2−/− mice, de novo erythropoiesis was impaired compared with wild-type mice, as shown by lesser numbers in total erythroid cells and the subpopulations of proerythroblasts and early and late erythroblasts (Fig. 7, A and B). Spleen histology showed red pulp expansion in hypoxic wild-type mice compared with normoxic controls, which is con-
JNK2 up-regulates HIF contributing to hypoxia pathobiology

consistent with a state of increased extramedullary erythropoiesis (46, 51, 52). However, we did not observe red pulp expansion in the spleen of Jnk2−/− mice (Fig. 7C). Similar results were obtained in the bone marrow. There were no differences in marrow erythropoiesis between wild-type and Jnk2−/− mice in the steady state (Fig. 7, D and E). Hypoxia treatment induced de novo erythropoiesis in the bone marrow of wild-type mice, as shown by increased numbers in total erythroid cells as well as in the subpopulations of proerythroblasts and early erythroblasts (Fig. 7, D and E). Again, under hypoxic conditions, Jnk2−/− mice exhibited less de novo erythropoiesis compared with wild-type mice, as shown by reduced numbers of total erythroid cells and subpopulations of proerythroblasts and early and late erythroblasts (Fig. 7, D and E). Taken together, these data suggest that JNK2 contributes to de novo erythropoiesis in response to hypoxia, both medullary and extramedullary. Considering the role of HIFs in hypoxia-induced erythropoiesis (53, 54), our in vivo data are consistent with our in vitro observation that JNK2 up-regulates HIFs (Figs. 1–3).
JNK2 up-regulates HIF contributing to hypoxia pathobiology

Jnk2−/− mice are protected from hypoxia-induced pulmonary hypertension

Our data suggest that JNK2 up-regulates HIF in vitro as well as in a mouse model of acute hypoxia-induced erythropoiesis. We sought to validate the regulation of HIF by JNK2 in vivo using a different mouse model of chronic hypoxia-induced PH. HIF-1α and HIF-2α are critical for the development of hypoxia-induced PH, as it has been reported that PH due to chronic hypoxia can be mitigated in either Hif-1α or Hif-2α heterozygous-null mice (15, 16). The positive regulation of HIFs by JNK2 prompted us to postulate that Jnk2−/− mice may be protected from chronic hypoxia-induced PH. Wild-type and Jnk2−/− mice were maintained at 10% oxygen for 21 days before hemodynamic measurements were obtained by directly measuring right ventricular systolic pressure using a micromanometer-tipped catheter. During these measurements, the mice were ventilated with 21% oxygen. As expected, Jnk2−/− mice were partially protected from hypoxia-induced PH, as shown by decreased right ventricular systolic pressures compared with wild-type mice (Fig. 8A). It is known that RBC content can influence viscosity, which in turn can lead to elevated pulmonary artery pressures. We measured the RBC count in the peripheral blood of wild-type and Jnk2−/− mice after chronic exposure to 10% oxygen for 21 days. Whereas RBC count in Jnk2−/− was decreased compared with wild-type mice under acute hypoxic conditions (7% oxygen for 7 days) (Fig. 6A), we observed no difference in the RBC count between these two groups of mice under chronic hypoxic conditions (10% oxygen for 21 days) (Fig. 8B). These data suggest that JNK2 is required to maintain de novo erythropoiesis under acute but not chronic hypoxic conditions, indicating that the hypoxia-induced erythropoietic response is only delayed and not diminished by the loss of JNK2. Furthermore, our data suggest that the reduction in the right ventricular systolic pressure observed in hypoxic Jnk2−/− mice compared with wild-type mice is not attributable to a lower vascular resistance caused by reduced erythropoiesis in hypoxia. It is interesting to note that Jnk2−/− mice recapitulate the phenotype of Hif-1α or Hif-2α heterozygous-null mice during hypoxic response, which also displayed a delayed erythropoietic response and reduced pulmonary hypertension (15, 16). Taken together, these data suggest that JNK2 deficiency protects mice from chronic hypoxia-induced PH, consistent with its role in HIF regulation.

Discussion

Hypoxia from any cause, whether from low barometric pressure, anaemia, or decreased alveolar-capillary diffusion, can have adverse effects on all organ systems and overall survival. As exemplified in chronic lung diseases, such as COPD, the presence of chronic hypoxia is associated with worse survival and quality of life and complications such as PH and polycythemia. In this context, the most commonly prescribed therapy is continuous oxygen supplementation (55), but disease progression and mortality are the norm. Here, we show that JNK2 mediates the up-regulation of HIF-1α and HIF-2α at the transcriptional level, which in turn contributes to the impact of hypoxia on erythropoiesis and PH. Thus, our data may provide a novel target for future therapies of hypoxia-associated diseases.

Our in vitro studies revealed that JNK2, but not JNK1, up-regulated HIF-1α and HIF-2α through increased mRNA expression rather than decreased post-translational degradation by the proteasome. In vivo, we observed decreased mRNA and protein levels of HIF-1α and HIF-2α in Jnk2−/− mice compared with wild-type mice under hypoxic conditions, which is consistent with our in vitro data. The in vivo relevance of JNK2 regulation of HIF was revealed by our observation that Jnk2−/− mice were alleviated from hypoxia-induced polycythemia and PH, two well-established phenotypes of overexpression of the HIF pathway. Thus, JNK2 may be an important mediator in the pathobiology of hypoxia-induced polycythemia and PH through the positive regulation of HIF-1α and HIF-2α expression.

A limited body of work has thus far implicated a role for JNK in the regulation of HIF and the hypoxic response in vitro (56–58). Comerford et al. (56) treated HeLa cells with hypoxia in the presence of a nonspecific JNK1/2/3 inhibitor and found decreased HIF-1α protein levels, decreased HIF-1 reporter activity, and decreased hypoxia response element binding. Subsequently, Zhang et al. (57) found that both JNK1 and JNK2 increased HIF-1α protein stabilization through molecular chaperone Hsp70/Hsp90 activity, JNK2 stabilized the HIF-1α mRNA transcripts by up-regulating nucleolin expression (58). Paradoxically, we did not observe that JNK2 stabilized either HIF-1α or HIF-2α mRNA in vitro. This discrepancy may be due to differences in

Figure 4. JNK2 up-regulates the expression of HIF-1α under pseudohypoxic conditions. A, HIF-1α immunoblot of wild-type or Jnk2−/− MEFs treated with DMOG (0.5 or 0.75 mM dissolved in DMSO) or vehicle control for 0 or 2 h. B, HIF-1α immunoblot of wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. C, quantitative PCR for relative HIF-1α mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DMOG (0.5 or 0.75 mM dissolved in DMSO) or vehicle control for 0 or 2 h. E, quantitative PCR for relative HIF-1α mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. F, quantitative PCR for relative HIF-1α mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. G, quantitative PCR for relative VEGF mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. H, quantitative PCR for relative iNOS mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. J, quantitative PCR for relative GLUT1 mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. J, quantitative PCR for relative GLUT1 mRNA expression after normalizing to HPRT in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. K, quantitative PCR for relative HIF-2α mRNA expression after normalizing to GLUT1 in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h. L, quantitative PCR for relative HIF-2α mRNA expression after normalizing to GLUT1 in wild-type or Jnk2−/− MEFs treated with DFO (100 mM dissolved in water) for 0 or 1 h.
the assay used; whereas Zhang et al. (57) used relative densitometry of agarose gel–separated PCR products after staining with ethidium bromide, we used quantitative RT-PCR for our measurements. Combined with our findings that JNK2 did not regulate HIF-1α or HIF-2α through regulation of their proteosomal degradation, we conclude that JNK2 regulation of HIF mRNA may be mediated through de novo mRNA transcription.

Figure 5. JNK2 up-regulates the expression of the HIF target gene Epo. A, JNK1 and JNK2 immunoblots from WT and Jnk2−/− mouse whole-lung homogenates. The blot shown is representative of the results of n = 4 in each genotype. B, quantitative PCR for relative HIF-1α mRNA expression after normalizing to HPRT in mouse kidney homogenates after 0, 1, 4, or 7 days of hypoxia (7% oxygen). n = 5 in control group, and n = 3 in each hypoxia group. C, quantitative PCR for relative HIF-2α mRNA expression after normalizing to HPRT in mouse kidney homogenates after 0, 1, 4, or 7 days of hypoxia (7% oxygen). n = 5 in control group, and n = 3 in each hypoxia group. D, HIF-1α immunoblot of whole-kidney homogenates from WT or Jnk2−/− mice kept in hypoxic conditions (7% oxygen) for 7 days. E, HIF-2α immuno blot of whole-kidney homogenates from WT or Jnk2−/− mice kept in hypoxic conditions (7% oxygen) for 7 days. F, HIF-1α immunoblot of whole-brain homogenates from WT or Jnk2−/− mice kept in normoxic or hypoxic conditions (7% oxygen) for 7 days. G, EPO levels in the serum of WT or Jnk2−/− mice kept in normoxic or hypoxic conditions (7% oxygen) for 7 days. Data are pooled from two independent experiments. n = 9 in the WT control group, n = 6 in the Jnk2−/− control group, n = 8 in WT hypoxia group, and n = 9 in Jnk2−/− hypoxia group. Immunoblots shown are representative of three independent experiments conducted with at least three mice in each group. All data are presented as mean ± S.E. (error bars). Statistical significance was determined by one-way ANOVA with Tukey’s post hoc test (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ns, non-significant for the comparisons indicated by brackets).
JNK2 up-regulates HIF contributing to hypoxia pathobiology
animal models of hypoxia-induced erythropoiesis and PH, we demonstrate that JNK2 is an important physiological contributor to the hypoxic response by maintaining HIF expression. Most in vitro studies indicate that HIF is predominantly regulated post-translationally through the inhibition of O2-dependent prolyl-4-hydroxylase domain proteins that drive HIF degradation under normoxia. Our results show that maintenance of HIF mRNA levels by JNK2, which precedes hypoxia-induced HIF protein accumulation, is of critical importance under pathophysiologically relevant conditions in vivo. Future studies are needed to investigate the molecular mechanisms by which JNK2 maintains HIF mRNA levels.

Several studies have shown that JNK is activated in response to hypoxia in a variety of biological systems to hypoxia, including the neurological (59–62), cardiovascular (63, 64), renal (65), hepatic (66), and oncological (67–69) systems. Specifically, Jin et al. (70) found that the activity of all MAPKs (JNK, ERK, and p38) was increased, with varying time courses, in the pulmonary arteries of hypoxia-treated rats. Our findings that mice deficient in JNK2 were relatively (but not completely) spared of hypoxia-induced polycythemia and PH are in line with these studies. The incomplete mitigation of the pulmonary hypertension by loss of JNK2 suggests contributions from additional factors.

In summary, we have demonstrated that the regulation of HIF-1α and HIF-2α by JNK2 is physiologically relevant in the hypoxic response. This regulation appears to occur by increasing HIF mRNA rather than through the inhibition of the canonical, post-translational ubiquitin–proteasomal degradation. Our data suggest that JNK2 may play a role in the development of hypoxia-induced polycythemia and PH, both common complications of chronic hypoxia in humans. Inhibition of JNK2 may be a novel therapeutic target for these conditions and possibly other HIF pathway-associated disease states.

**Experimental procedures**

**Animal model of hypoxia**

Wild-type or Jnk2−/− C57BL/6 mice (at least 8 weeks old) were treated with normobaric normoxia (21% oxygen) or in an enclosed normobaric hypoxia chamber (7% oxygen) for 1, 4, or 7 days (acute hypoxia model) or 10% oxygen for 21 days (chronic hypoxia model). Post-hypoxia, mice were weighed and then sacrificed with Euthasol euthanizing solution. Peripheral blood was collected directly into EDTA-lined tubes to proceed to automated cell counting using a Hemavet 950FS system (Drew Scientific, Inc.). For a separate subgroup of mice, spleens and femoral bone marrow were collected and made into single cell suspensions for flow cytometry (see below). For another subgroup of mice, spleens were fixed, embedded in paraffin, and analyzed by staining with hematoxylin and eosin. The animal care and experiments were performed in compliance with institutional and National Institutes of Health guidelines and were approved by the Northwestern University Animal Care and Use Committee.

**Cell culture**

Wild-type, Jnk1−/−, and Jnk2−/− MEFs have been described (25, 33). Cells were incubated at 37 °C in an atmosphere of 21% oxygen, 5% CO2, before experimental exposures. Cells were maintained according to ATCC (Manassas, VA) recommendations.

**EPO analysis**

The concentration of erythropoietin in the mouse serum was measured using multiplex immunoassay according to the manufacturer’s instructions (eBioscience).

**In vitro model of hypoxia**

Cells were exposed to defined atmospheric conditions in environmental chambers (InvivO2 hypoxia workstation, Baker Co.). Normoxia was defined as 21% oxygen and 5% carbon dioxide, and hypoxia was defined as 1.5% oxygen and 5% carbon dioxide. Cell lysates were harvested immediately after the treatment course was completed. Starvation medium (2.5% FBS, 2.0% HEPES) was used for all hypoxia experiments and was pre-equilibrated overnight in the hypoxic atmosphere. For the MG132 experiments, cells were treated with 20 μM MG132 (dissolved in DMSO) 2 h before hypoxia exposure. For the echinomycin experiments, cells were treated with 10 nm echinomycin (dissolved in DMSO) (SMLO477, Sigma-Aldrich) for 1 h before hypoxia exposure. For siRNA transfection experiments, the following siRNA oligonucleotides were from Thermo Scientific (Dharmacon products): control siRNA (D-001210-02), JNK1-specific siRNA (5′-UGAUUCAGAUGGAGUUAGATT-3′), and JNK2-specific siRNA (5′-CCGCAGAGUUCAUGAAGAAT-3′). HIF-1α-specific siRNA (NM_010431) was from Sigma-Aldrich.

**Western blotting**

Protein content was normalized with spectrophotometry and subsequently resolved on 10% polyacrylamide gels. Separated proteins were then transferred to PVDF membranes and incubated overnight with primary antibodies. The following primary antibodies were used: HIF-1α (10006421, 1:200; Cayan, Zymed Chemical, Ann Arbor, MI), HIF-2α (ab199, 1:500; Abcam), JNK2 (4672, 1:500; Cell Signaling Technology, Beverly, MA), JNK1/JNK2 (G151-666, 1:50; BD Biosciences), and β-actin (A5441, 1:20,000; Sigma-Aldrich).

Figure 6. Jnk2−/− mice have decreased RBC count, reticulocytosis, and splenomegaly under hypoxic conditions. A, red blood cell counts from WT or Jnk2−/− mice exposed to hypoxia (7% oxygen) for 7 days or kept in normoxic conditions. Data are pooled from five independent experiments. n = 19 in WT control group, n = 18 in Jnk2−/− control group, n = 50 in WT hypoxia group, and n = 37 in Jnk2−/− hypoxia group. B, reticulocyte percentage manually counted after staining with methylene blue dye. n = 2 in each control group, and n = 3 in each hypoxia (7% oxygen) group (a total of 500 cells were counted for each smear). C, representative reticulocyte stain with methylene blue dye of peripheral blood smears from WT or Jnk2−/− mice kept in normoxic conditions or treated with hypoxia (7% oxygen) for 7 days at ×40 magnification. Red arrows, reticulocytes. Images shown are representative of smears obtained from two independent experiments. D, representative photo of dissected spleens from WT or Jnk2−/− treated with hypoxia for 0, 4, or 7 days. E, spleen weight normalized to body weight of WT or Jnk2−/− mice treated with hypoxia (7% oxygen) for 7 days or kept in normoxic conditions. Data are pooled from five independent experiments. n = 16 in WT control group, n = 14 in Jnk2−/− control group, n = 52 in WT hypoxia group, and n = 39 in Jnk2−/− hypoxia group. All data are presented as mean ± S.E. (error bars). Statistical significance was determined by one-way ANOVA with Tukey’s post hoc test (**, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001; ns, non-significant for the comparisons indicated by brackets).
JNK2 up-regulates HIF contributing to hypoxia pathobiology

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**JNK2 up-regulates HIF contributing to hypoxia pathobiology**

**Flow cytometry**

Spleens were enzymatically digested by instillation with a mixture of DNase I and collagenase D and then passed through a 40-μm filter to obtain single cell suspension. 2.5 × 10⁶ cells were stained with Fixable viability dye, phycoerythrin-conjugated anti-Ter119 (12-9291-83, eBioscience), and allophycocyanin-conjugated anti-CD71 (17-0711-82, eBioscience). Femoral bone marrow was isolated as single cell suspension by passing through a 40-μm filter, and 2.5 × 10⁶ cells were stained with the same fluorochromes. All data collection and sorting were performed using BD FACS Diva software (BD Biosciences), and data analyses were performed using FlowJo software (Tree Star). Compensation matrices were calculated and applied using FlowJo software (Tree Star).

**Quantitative real-time PCR**

Total RNA was isolated from MEFS or homogenized kidneys using TRIzol reagent (Invitrogen). RNA was converted into cDNA using M-MuLV reverse transcriptase, and mRNA abundance was quantified by quantitative PCR using SYBR Green (Bio-Rad) and normalized to HPRT for the following primers: HIF-1α (forward, 5′-ATGAGTTCTGAACGTGAAAGA-3′; reverse, 5′-GGGGAAGTCGAACATGATGA-3′), HIF-2α (forward, 5′-CTGGACAAAGCCTCCATCAT-3′; reverse, 5′-TTGCTGATGTTTTCCGACAG-3′), JNK1 (forward, 5′-GTTCGCCGATGTGCTTTCC-3′; reverse, 5′-GGTGCTGGAGACCTCATCT-3′), JNK2 (forward, 5′-AGGTGCGACACTCACTTTTC-3′; reverse, 5′-CGAGTTCACGTTGCTCTCTTCTT-3′), iNOS (forward, 5′-AGGGCCACCTCTACATTAGGT-3′; reverse, 5′-GTGCCAGAAGCTGACTCTTT-3′), VEGF (forward, 5′-CCACGTCAAGAGCAACATACT-3′; reverse, 5′-TCATTCTCTATGTGCTGGTTTT-3′), GLUT1 (forward, 5′-CATCTTTATTGCCCAGGTTTT-3′; reverse, 5′-GAAGACGACACTTGAGGAGCAGA-3′), and HPRT (forward, 5′-AGCCACACTGAGCAGGCA-3′). Data are summarized as mean ± S.E. for n independent experiments. Statistical significance was assessed using a t test or one-way ANOVA followed by the appropriate post test. Within the figures, levels of statistical significance are denoted as follows: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001; ns, non-significant.

**Right ventricular systolic pressure measurements**

Mice were housed in normobaric normoxia (21% oxygen) or chronic hypoxia (10% oxygen) conditions for 21 days. Pulmonary artery pressure was quantified by right heart catheterization with a micromanometer-tipped catheter while being ventilated at 21% oxygen (Millar Instruments, Houston, TX) using a modification of a previous technique (17, 71) before euthanasia.

**Statistical analysis**

Data are summarized as mean ± S.E. for n independent experiments. Statistical significance was assessed using a t test or one-way ANOVA followed by the appropriate post test. Within the figures, levels of statistical significance are denoted as follows: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001; ns, non-significant.

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**Figure 7. Jnk2−/− mice have decreased splenic erythropoiesis under hypoxic conditions.** A, representative flow cytometry gating strategy for mouse spleen erythroid precursors. Quadrants were used to divide the cell populations into four groups representing progressive erythroid precursor differentiation (see "Results"). B, absolute counts of erythroid precursors in the spleen measured by flow cytometry in WT or Jnk2−/− mice after treatment with 7 days of hypoxia (7% oxygen) or kept in normoxic conditions. Data are pooled from two independent experiments, n = 5 in WT control group and n = 5 in Jnk2−/− control group, n = 5 in WT hypoxia group, and n = 9 in Jnk2−/− hypoxia group. C, representative H&E-stained sections of WT or Jnk2−/− mice spleens treated with hypoxia (7% oxygen) for 7 days or kept in normoxic conditions (×10 or ×40 magnification). D, representative flow cytometry gating strategy for mouse bone marrow erythroid precursors. Quadrants were used to divide the cell populations into four groups representing progressive erythroid precursor differentiation (see "Results"). E, absolute counts of erythroid precursors in the bone marrow measured by flow cytometry in WT or Jnk2−/− mice after treatment with 7 days of hypoxia (7% oxygen) or kept in normoxic conditions. Data are pooled from two independent experiments, n = 5 in WT control group and n = 5 in Jnk2−/− control group, n = 5 in WT hypoxia group, n = 9 in Jnk2−/− hypoxia group. N, normoxia; H, hypoxia; RBC, red blood cell content calculated as the sum of the areas of quadrants I, II, and III. All data are presented as mean ± S.E. (error bars). Statistical significance was determined by one-way ANOVA with Tukey’s post hoc test (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001; ns, non-significant for the comparisons indicated by brackets).

**Figure 8. Jnk2−/− mice are protected from hypoxia-induced pulmonary hypertension.** A, right ventricular systolic pressure measurements in WT or Jnk2−/− mice kept in normoxic conditions (21% oxygen) or chronic hypoxic conditions (10% oxygen) for 21 days. B, red blood cell count in the peripheral blood of WT or Jnk2−/− mice kept at 10% oxygen for 21 days. n = 5 in each group. All data are presented as mean ± S.E. (error bars). Statistical significance was determined in A by one-way ANOVA with Tukey’s post hoc test and in B by a t test (*, p ≤ 0.05; ***, p ≤ 0.0001; ns, non-significant for the comparisons indicated by brackets).
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