LETTERS

NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α

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The hypoxic response is an ancient stress response triggered by low ambient oxygen (O_2) (ref. 1) and controlled by hypoxia-inducible transcription factor-1 (HIF-1), whose α subunit is rapidly degraded under normoxia but stabilized when O_2-dependent prolyl hydroxylases (PHDs) that target its O_2-dependent degradation domain are inhibited[2–4]. Thus, the amount of HIF-1α, which controls genes involved in energy metabolism and angiogenesis, is regulated post-translationally. Another ancient stress response is the innate immune response, regulated by several transcription factors, among which NF-κB plays a central role[5,6]. NF-κB activation is controlled by IκB kinases (IKK), mainly IKK-β, needed for phosphorylation-induced degradation of IκB inhibitors in response to infection and inflammation[7]. IKK-β is modestly activated in hypoxic cell cultures when PHDs that attenuate its activation are inhibited[8]. However, defining the relationship between NF-κB and HIF-1α has proven elusive. Using *in vitro* systems, it was reported that HIF-1α activates NF-κB[9], that NF-κB controls HIF-1α transcription[10] and that HIF-1α activation may be concurrent with inhibition of NF-κB[11]. Here we show, with the use of mice lacking IKK-β in different cell types, that NF-κB is a critical transcriptional activator of HIF-1α and that basal NF-κB activity is required for HIF-1α protein accumulation under hypoxia in cultured cells and in the liver and brain of hypoxic animals. IKK-β deficiency results in defective induction of HIF-1α target genes including vascular endothelial growth factor. IKK-β is also essential for HIF-1α accumulation in macrophages experiencing a bacterial infection. Hence, IKK-β is an important physiological contributor to the hypoxic response, linking it to innate immunity and inflammation.

Hypoxia is characterized by a decreased O_2 tension within cells and can occur under several pathophysiological situations including ischaemia, cancer and inflammation[12]. During ischaemia, the flow of nutrients and O_2 to damaged tissues is decreased and HIF-1α activation induces genes whose products restore blood supply, nutrients and energy production, thereby maintaining tissue integrity and homeostasis[13,14]. The hypoxic response is important for the proper function of tissue macrophages and infiltrating neutrophils that encounter low O_2 tension in infected tissues and after bacterial replication[15]. HIF-1α was also suggested to promote the expression of inflammatory cytokines, which are known to be regulated by NF-κB[16], in lipopolysaccharide (LPS)-stimulated macrophages[17] and mediate NF-κB activation in anoxic neutrophils[9]. However, it was also reported that hypoxia leads to modest IKK-β activation by inhibiting PHDs that negatively modulate IKK-β activity[8]. We therefore decided to critically explore the relationship between IKK-β, NF-κB and HIF-1α under *in vivo* conditions in IKK-β-deficient mice and primary macrophages.

We first examined bone marrow-derived macrophages (BMDM) from either $IKK\beta^{F/F}$ or $IKK\beta^{F/F}$/*Mx1Cre* mice challenged with poly(I)•poly(C), which induces interferon (IFN) and thereby drives CRE recombinase expression from the *Mx1* promoter to delete *IKKβ* in IFN-responsive cells of the resulting $IKK\beta^{\Delta}$ mice[18]. BMDM were incubated with Gram-positive (group A *Streptococcus*; GAS) and with Gram-negative (*Pseudomonas aeruginosa*) bacteria. Both species induced HIF-1α accumulation in an IKK-β-dependent manner (Fig. 1a). The induction of HIF-1 target genes involved in the hypoxic and innate immune responses was also dependent on IKK-β (Fig. 1b). These genes included *Cox-2*, which is directly regulated by NF-κB and HIF-1α, *Cnlp*, which encodes the murine antimicrobial peptide mCRAMP, whose expression is not directly responsive to NF-κB[19], and *Glut-1*, encoding a glucose transporter. Moreover, *Hif1a* mRNA was markedly downregulated in IKK-β-deficient cells even before infection (Fig. 1b). IκB degradation and the nuclear accumulation of RelA/NF-κB preceded HIF-1α expression (Fig. 1c), indicating that NF-κB may control *Hif1a* gene transcription. Indeed, chromatin immunoprecipitation (ChIP) in LPS-stimulated macrophages revealed that RelA is recruited to the *Hif1a* promoter, which contains a classical κB site at −197/−188 base pairs, conserved between mice and humans (Fig. 1d). Furthermore, the basal levels of *Hif1a* mRNA were decreased in RelA-deficient fibroblasts even under resting conditions (Supplementary Fig. 1), suggesting that NF-κB activity is required for effective *Hif1a* mRNA expression even in non-stimulated cells.

As found elsewhere[8], hypoxia modestly activated IKK in macrophages (Fig. 2a), induced the phosphorylation of IKK-α/β and IκBα and promoted IκBα degradation (Fig. 2b). Hypoxia also induced the nuclear translocation of RelA, which preceded HIF-1α accumulation (Fig. 2c), as occurred in bacteria-infected macrophages (Fig. 1c). Binding of NF-κB to a canonical κB DNA site was also induced by hypoxia (Fig. 2d). We examined whether IKK-β was required for hypoxia-induced HIF-1α accumulation, a response that is thought to be dependent mainly on inhibition of HIF-1α degradation[3,4]. IKK-β was required for the optimal accumulation of HIF-1α, but not of HIF-2α, in BMDM incubated with the hypoxia mimetic desferrioxamine (DFX) as well as in response to actual hypoxia (Fig. 3a, b). IKK-β also did not affect HIF-2α expression in infected macrophages (Fig. 1a). The overexpression of a non-degradable IκBα (IκB superrepressor) also blocked HIF-1α accumulation induced by hypoxia in HEK-293 cells (Supplementary Fig. 2). The hypoxia-dependent induction of HIF-1 target genes, such as those encoding vascular endothelial growth factor (VEGF) and GLUT-1, was nearly abolished in IKK-β-deficient macrophages (Fig. 3c) or fibroblasts (Supplementary Fig. 3). Expression of *Hif1a*, but not *Hif2α*, mRNA was

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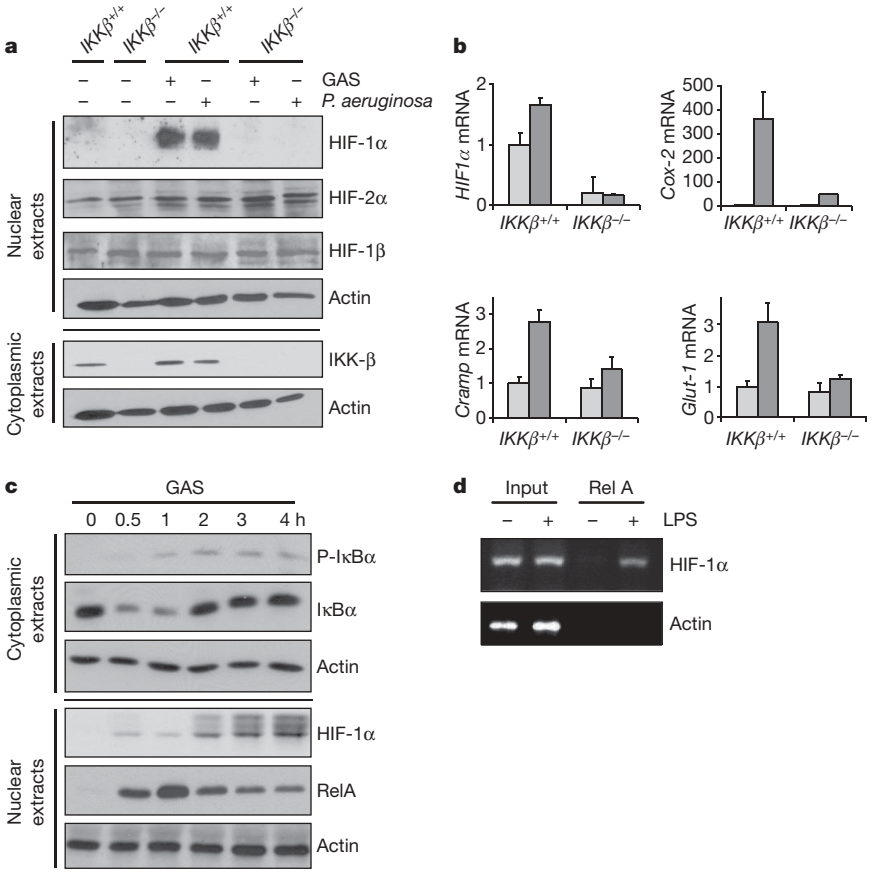

Figure 1 | IKK-β is required for microbial-induced HIF-1α expression in macrophages. **a**, BMDM from either $IKK\beta^{F/F}$ ($IKK\beta^{+/+}$) or poly(I)•poly(C)-injected $IKK\beta^{F/F}$/*Mx1-Cre* ($IKK\beta^{\Delta}$; $IKK\beta^{-/-}$) mice were incubated with either with GAS or *P. aeruginosa* (MOI of 10 for 4 h). Expression of the indicated proteins was analysed by immunoblotting. **b**, RNA was extracted from BMDM incubated with GAS and gene expression was analysed by quantitative RT–PCR. Results are averages of three separate experiments done in triplicate, and are shown as means and s.e.m. Values are normalized relative to 18S rRNA. Light grey bars, control; dark grey bars, GAS. *Cramp*, gene encoding cathelicidin antimicrobial peptide. **c**, RAW264.7 macrophages were incubated with GAS and protein expression was analysed by immunoblotting at the indicated time points. **d**, ChIP was performed with an anti-RelA antibody using fixed and sheared chromatin isolated from RAW264.7 mouse macrophages incubated for 1 h with or without LPS. The *Hif1a* promoter fragment, which contains a κB site at −197/−188 base pairs, was detected by PCR amplification.

substantially decreased in the absence of IKK-β even under normoxia (Fig. 3c), further supporting the notion that basal NF-κB activity is required for the expression of enough *Hif1a* mRNA at all times to result in the rapid accumulation of HIF-1α protein, which occurs only under hypoxic conditions. Activation of NF-κB by LPS induced *Hif1a* promoter activity (Supplementary Fig. 4), elevated HIF-1α expression in hypoxic cells (Fig. 3d) and potentiated the induction of *Vegf* mRNA (Supplementary Fig. 5). Despite substantial expression of *Hif1a* mRNA in LPS-stimulated normoxic macrophages (Supplementary Fig. 5), these cells do not accumulate HIF-1α protein (Fig. 3d), which echoes findings in T cells stimulated with anti-CD3 antibody[20]. Hence, NF-κB activation without hypoxic inhibition of PHDs is insufficient for HIF-1α protein accumulation. In mouse fibroblasts, IKK-β was required for basal *Hif1a* promoter activity and its stimulation by treatment with DFX (Fig. 3e).

We next examined the role of IKK-β in HIF-1 activation in intact mice. Administration of DFX induced HIF-1α expression in liver of $IKK\beta^{F/F}$ mice but not in $IKK\beta^{\Delta}$ mice (Fig. 4a), which lack IKK-β in both hepatocytes and Kupffer cells[21]. $IKK\beta^{\Delta}$ mice also contained less *Hif1a* and *Vegf* mRNA in their livers (Fig. 4b). We also examined the role of IKK-β in the response to actual hypoxia. Mice were placed in a chamber with an ambient O_2 concentration of 8% (thus mimicking an altitude of 7,000 m (ref. 22)). Under these conditions we observed hypoxia-induced HIF-1α accumulation in liver (Fig. 4c) and brain (Fig. 4d) and in both cases it was dependent on IKK-β in CRE-expressing cells. In the brain the predominant CRE-expressing cells were astrocytes (Supplementary Fig. 6) and not neurons (data not shown), thus explaining the partial deletion of IKK-β in this tissue (Fig. 4d). Despite this, hypoxia-induced VEGF protein (Fig. 4e) and *Vegf* mRNA (Fig. 4f) were IKK-β dependent. $IKK\beta^{\Delta}$ mice showed a profound increase in cerebellar astrocyte activation, marked by glial fibrillary acidic protein, relative to $IKK\beta^{F/F}$ mice (Supplementary Fig. 7). This may have been due to defective production of VEGF, a cytokine with anti-inflammatory properties that has been shown to promote tissue repair[23]. VEGF is also a potent neuroprotective factor[24] whose decreased production may potentiate hypoxia-induced neuronal damage and thereby augment astrocyte activation. This situation may be akin to a loss of IKK-β in intestinal epithelial cells, which has previously been found to exacerbate ischaemic damage to the intestinal mucosa[25]. These results suggest that IKK-β inhibitors may not be useful in the treatment of neuroinflammatory disorders.

Although early studies demonstrated the induction of *Hif1a* mRNA in experimental animals during development and hypoxia[26,27], numerous *in vitro* studies led to the current model that the accumulation of HIF-1α is regulated predominantly at the post-translational level through the inhibition of O_2-dependent PHDs that drive HIF-1α degradation under normoxia[3,4]. Our results show clearly that transcriptional activation of the *Hif1a* gene by IKK-β-responsive NF-κB, which precedes HIF-1α protein accumulation, is of critical importance under pathophysiologically relevant conditions *ex vivo* and *in vivo*. Both macrophages infected with bacteria and mice subjected to hypoxia reveal a pronounced defect in HIF-1α expression on loss of

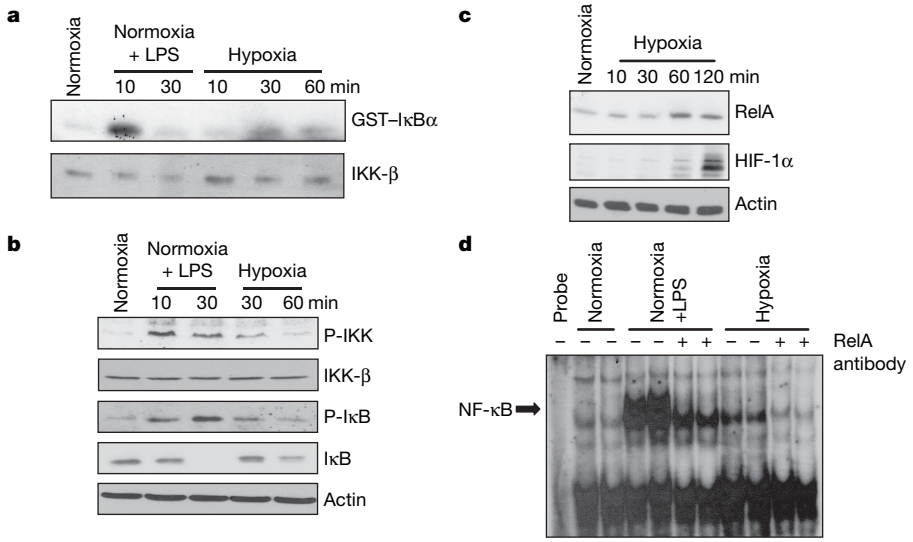

Figure 2 | Hypoxia activates the NF-kB pathway in macrophages. RAW264.7 macrophages were incubated with or without LPS under normoxia or were placed under hypoxia (0.5% O_2). **a**, At the indicated time points, IKK activity was measured by an immunocomplex kinase assay with GST–IκBα as a substrate. **b**, Cell lysates were prepared and IKK-β and IκBα phosphorylation (P) and amounts were analysed by immunoblotting. **c**, Nuclear extracts were prepared at the indicated time points and analysed by immunoblotting for the nuclear accumulation of RelA and HIF-1α. **d**, Nuclear extracts were prepared after 2 h of stimulation with LPS or hypoxia, and binding activity for DNA for NF-κB was examined by a mobility-shift assay. Antibody inhibition was performed with an anti-RelA antibody.

IKK-β. The IKK-β/NF-κB–HIF-1α crosstalk is not critical during normal embryonic development, because the respective gene deletions result in different phenotypes. Whereas *Hif1a*$^{-/-}$ embryos die prematurely at embryonic day 9.5, mainly as a result of defects in neural fold closure and capillary development[13,14], *IKK-β*$^{-/-}$ embryos die later, at embryonic day 13.5, from massive liver apoptosis driven by TNF[28,29].

Previous findings identified a connection between HIF-1α and innate immunity and inflammation, but it was not clear how microbial infection or inflammation led to HIF-1α activation[15,19]. Our

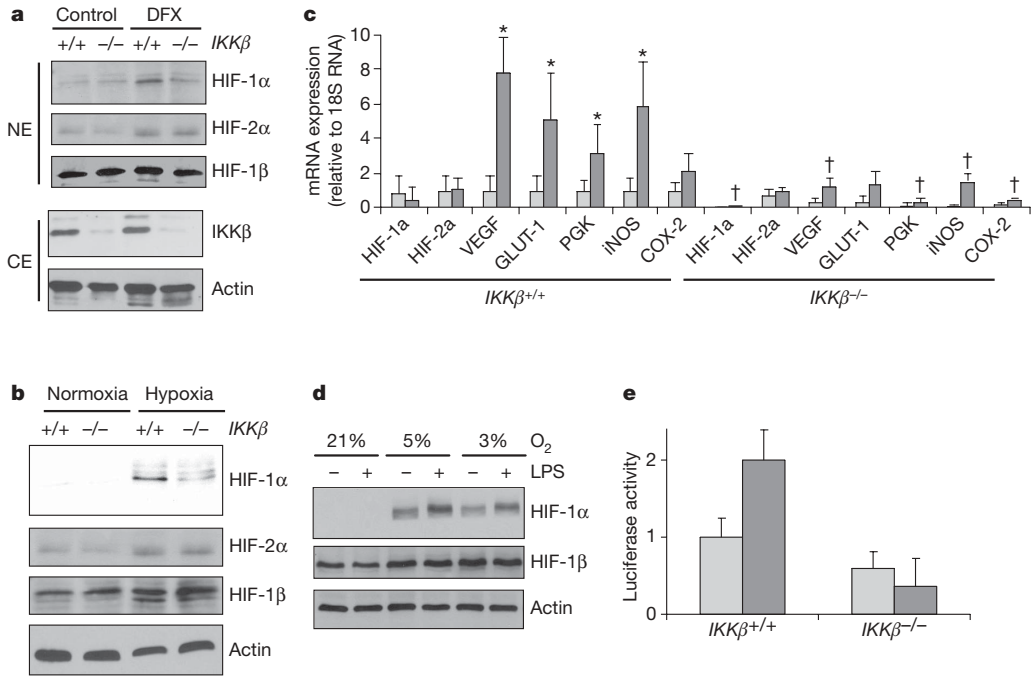

Figure 3 | IKK-β regulates hypoxia-induced HIF-1α and target genes in mouse macrophages. **a**, BMDM from *IKKβ*$^{F/F}$ (IKK-β$^{+/+}$) or *IKKβ*^{Δ} (IKK-β$^{-/-}$) mice were incubated with DFX for 4 h. Expression of the indicated proteins was analysed by immunoblotting for nuclear (NE) and cytosolic (CE) extracts. **b**, BMDM were obtained as above and cultured under normoxia or hypoxia (0.5% O_2 for 4 h). Protein expression was analysed by immunoblotting. **c**, BMDM were treated as above and mRNA expression was analysed by quantitative RT–PCR. Light grey bars, normoxia; dark grey bars, hypoxia. Results are means and s.e.m. for three separate experiments performed in triplicate. *, $P < 0.05$ versus normoxic *IKKβ*$^{+/+}$ cells; †, $P < 0.05$ versus hypoxic *IKKβ*$^{+/+}$ cells. PGK, phosphoglucokinase; iNOS, inducible nitric oxide synthase. **d**, RAW264.7 macrophages were cultured in the absence or presence of LPS under the indicated O_2 tensions for 2 h. Protein expression was analysed by immunoblotting. **e**, Murine embryonic fibroblasts from *IKKβ*$^{+/+}$ or *IKKβ*$^{-/-}$ embryos were transfected with a luciferase reporter gene driven by the *Hif1a* promoter. After 36 h the cells were incubated for 3 h with DFX. Light grey bars, control; dark grey bars, DFX. Results are means and s.e.m. for three separate experiments performed in triplicate.

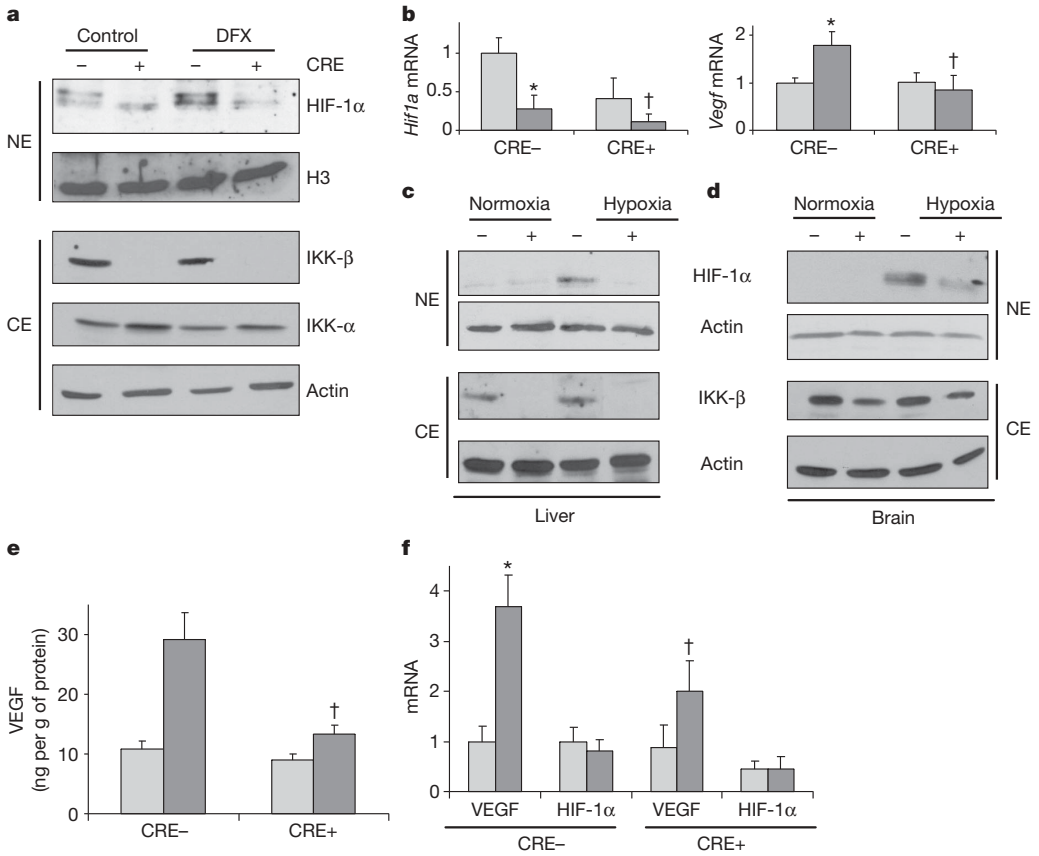

Figure 4 | IKK-β regulates HIF-1α expression in hypoxic mice. $IKK\beta^{F/F}$ (CRE−) or $IKK\beta^{\Delta}$ (CRE+) mice were treated with vehicle (control) or DFX (600 mg kg^{-1}). After 15 h, livers were removed for protein (**a**) and RNA (**b**) analysis. **a**, HIF-1α and IKK-β expression in nuclear (NE) or cytosolic (CE) extracts was analysed by immunoblotting. **b**, Expression of HIF-1α and *Vegf* mRNA was examined by quantitative RT–PCR. Light grey bars, control; dark grey bars, DFX. Results are means and s.e.m. ($n = 3$). Values are normalized relative to 18S rRNA. *, $P < 0.05$ versus normoxic CRE− mice; †, $P < 0.05$ versus DFX-treated CRE− mice. **c**, **d**, $IKK\beta^{F/F}$ and $IKK\beta^{\Delta}$ mice were kept under normoxia or hypoxia (8% O_2) for 24 h and HIF-1α and IKK-β expression was analysed by immunoblotting of liver (**c**) or brain (**d**) nuclear and cytosolic extracts, respectively. **e**, VEGF expression in brain of mice from the above experiment was analysed by ELISA. Light grey bars, normoxia; dark grey bars, hypoxia. Results are means and s.e.m. ($n = 3$). *, $P < 0.05$ versus normoxic CRE− mice; †, $P < 0.05$ versus hypoxic CRE− mice. **f**, VEGF and *Hif1a* mRNA expression were analysed by quantitative RT–PCR of total brain RNA. Light grey bars, normoxia; dark grey bars, hypoxia. Results are means and s.e.m. *, $P < 0.05$ versus normoxic CRE− mice; †, $P < 0.05$ versus hypoxic CRE− mice ($n = 3$).

results, together with the previous finding that IKK-β catalytic activity is controlled by O_2-sensitive PHDs[8], establish NF-κB as a hypoxia-regulated transcription factor that controls *Hif1a* mRNA expression both under basal conditions and during hypoxia, thereby serving as a regulator of the hypoxic response. Our findings demonstrate that this depends on NF-κB activation, which controls *Hif1a* mRNA expression, but accumulation of HIF-1α protein requires hypoxia, which in bacterial infection may be due to depletion of intracellular oxygen by replicating bacteria. These findings have far-reaching physiological implications because they indicate the existence of coupling between two evolutionary ancient stress responses: innate immunity and the hypoxic response. By controlling HIF-1α activation in macrophages during microbial infections, which may lower local O_2 tension, NF-κB can enhance glycolytic energy metabolism and the production of angiogenic factors, in addition to its well-established role in the expression of proinflammatory cytokines, chemokines and antimicrobial peptides. In addition to more effective execution of the host-defence response, the ability of NF-κB to promote HIF-1α activation expands its pro-survival function because the HIF-1-dependent hypoxic response is critical for providing cells and tissues undergoing ischaemia with sufficient energy supplies and allows them to resist cell death.

By serving as an essential component of the hypoxic response *in vivo*, IKK-β also performs a homeostatic function in the brain, an organ that is extremely sensitive to deprivation of oxygen and glucose[30].

METHODS SUMMARY

To delete IKK-β in $IKK\beta^{F/F}$/*Mx1Cre* mice, 250 μg of poly(I)•poly(C) (Sigma) was injected intraperitoneally on three alternate days, three weeks before exposure to hypoxia or isolation of myeloid cells[18]. To induce hypoxia *in vivo*, mice were placed in a special chamber in which N_2 and O_2 were injected to achieve an O_2 concentration of 8 ± 0.1%. This was controlled by the Oxycycler hydraulic system (Model A44x0; BioSpherix) and ANA-Win2 software (Version 2.4.17; Watlow Anafaze). Control mice were kept in the same room under normal atmospheric O_2 and were exposed to the same level of noise and light during each experiment. After 24 h of normoxia or hypoxia, mice were killed and their livers and brains were rapidly removed and frozen in liquid N_2 or OCT with a solid CO_2/2-methylpropan-1-ol bath.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Quantitative RT–PCR. Total RNA was extracted with Trizol (Invitrogen) and reverse-transcribed with random hexamers and SuperScript II Kit (Invitrogen). Real-time PCR was performed with SYBR Green PCR Master Mix Kit (Applied Biosystems). The following primer pairs were used: VEGF, 5′-CCACGTCAGAGAGCAACATCA-3′ and 5′-TCATTCTCTCTATGTGCTGGCTTT-3′; PGK, 5′-GGAAGCGGGTCGTGATGA-3′ and 5′-GCCTTGATCCTTTGGTTGTTTG-3′; GLUT-1, 5′-CATCCTTATTGCCCAGGTGTTT-3′ and 5′-GAAGACGACACTGAGCAGCAGA-3′; iNOS, 5′-GGCAGCCTGTGAGACCTTTG-3′ and 5′-CATTGGAAGTGAAGCGTTTCG-3′; COX-2, 5′-GTGGAAAAACCTCGTCCAGA-3′ and 5′- GCTCGGCTTCCAGTATTGAG-3′; HIF-1α, 5′-ACAAGTCACCACAGGACAG-3′ and 5′-AGGGAGAAAATCAAGTCG-3′; HIF-2α, 5′-CAACCTGCAGCCTCAGTGTATC-3′ and 5′-CACCACGTCGTTCTTCTCGAT-3′; 18S rRNA, 5′-CGCCGCTAGAGGTGAAATTCT-3′ and 5′- CGAACCTCCGACTTTCGTTCT-3′.

Immunoblotting. Whole-cell extracts were obtained by lysing cells in 1% SDS, 10 mM Tris-HCl pH 7.4. Cytoplasmic and nuclear extracts were obtained as described[2]. Proteins were separated by SDS–PAGE and detected by immunoblotting. Blots were incubated with antibodies against phosphorylated IKK-α/β, phosphorylated IκBα, IKK-α, IKK-β, IκBα, RelA and histone H3 (all from Santa Cruz Biotechnology), actin (Sigma), HIF-1α, HIF-2α and HIF-1β (Novus).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed with ChIP-IT Express Kit (Active Motif) in accordance with the manufacturer's instructions. Chromatin was precipitated with RelA antibodies (Santa Cruz Biotechnology). Samples were analysed by PCR. The murine HIF-1α and actin promoters were amplified with the primer pairs 5′-CACCCCCATCTCCTTTCTCT-3′ and 5′-GGGTTCCTCGAGATCCAATG-3′, and 5′-TGCACTGTGCGGCGAAGC-3′ and 5′-TCGAGCCATAAAAGGCAA-3′, respectively.

Luciferase assay. A murine HIF-1α-luciferase reporter, pHIF-1α/Luc, was kindly provided by S. W. Ebbinghaus. pHIF-1α/Luc was co-transfected with the internal control pRL-TK into either $IKK\beta^{+/+}$ or $IKK\beta^{-/-}$ MEFs with Lipofectamine 2000 (Invitrogen). Luciferase activity was measured with the Dual-luciferase reporter assay system (Promega). Results are presented as relative reporter activity after normalization to the internal control pRL-TK.

Statistical analysis. Results are expressed as means and s.e.m. A Stat View II (Abacus Concepts) statistical package was used for all analyses: multiple groups were compared by one-factor analysis of variance, followed by Fisher's protected least-squares difference to assess specific group differences.