



TNF α induces HIF-1 α expression through activation of IKK β

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ABSTRACT

The transcription factor hypoxia-inducible factor 1 α (HIF-1 α) is regulated by oxygen availability as well as various inflammatory mediators, including tumor necrosis factor α (TNF α). Early work suggested that the phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways are involved in TNF α -mediated HIF-1 α accumulation and activation under normoxic conditions. Here, we provide evidence showing that I κ B kinase β (IKK β) is required for HIF-1 α regulation by TNF α . We found that TNF α enhances HIF-1 α protein expression in various breast cancer cell lines under either normoxic or hypoxia-mimicking conditions, but has little effect on the HIF-1 α mRNA level. Increased HIF-1 α expression was found in IKK β stable clones and transient transfectants, and depletion of IKK β consistently reduced the amount of HIF-1 α protein. Treatment of cells with the IKK β inhibitor Bay 11-7082 reduced the TNF α -induced HIF-1 α expression, suggesting that IKK β is required in this signaling pathway. Decreased expression of vascular endothelial growth factor (VEGF), a direct target of HIF-1 α , was shown in IKK β -knockout mouse embryonic fibroblast cells. We further demonstrated a positive correlation between IKK β and VEGF expression in primary human breast cancer specimens. Our findings indicate that TNF α -induced HIF-1 α accumulation is IKK β dependent, and may enable further understanding of the HIF-1 α regulation by inflammatory signals.

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Introduction

Hypoxia-inducible factor 1 α (HIF-1 α), a critical transcription factor, coordinates physiological responses to low oxygen availability. Under hypoxic conditions, HIF-1 α translocates into the nucleus and forms heterodimers with aryl hydrocarbon receptor nuclear translocator (ARNT, HIF-1 β), which then bind to hypoxic response elements (HREs) in the promoter/enhancer region of target genes [1]. This results in the transcription of various genes involved in critical cellular functions, including energy metabolism, angiogenesis, erythropoiesis, and apoptosis [2–4]. The angiogenic factor vascular endothelial growth factor (VEGF), which stimulates angiogenesis and increases the permeability of blood vessels in the local area, is one of the direct target genes of HIF-1 α .

Although under the current paradigm, HIF-1 α is a prominent regulator of the response to hypoxia, it is now appreciated that the expression and activation of HIF-1 α can also be induced by the inflammation process [5,6]. Pro-inflammatory cytokines such

as tumor necrosis factor α (TNF α) activates HIF-1 α even under normoxic conditions [5]. Several intracellular signaling pathways, including phosphatidylinositol-3-kinase (PI3K)–AKT, and mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinase (ERK), are known to be stimulated by TNF α and involved in TNF α -mediated HIF-1 α activation. While activation of the PI3K–AKT pathway causes an increase in the HIF-1 α protein level [7,8], activation of the MAPK–ERK pathway mainly enhances the transactivation potential of HIF-1 α [9,10].

Along with AKT and ERK, I κ B kinase β (IKK β) is an oncogenic kinase that is frequently activated in many human cancers. Growing evidence has shown that these three kinases function in common signaling pathways to exert their oncogenicity such as by inactivation of the tumor suppressors forkhead box O3a (FOXO3a) and tuberous sclerosis complex (TSC) through phosphorylation. AKT, ERK, and IKK β phosphorylate FOXO3a at different sites, resulting in its translocation and degradation [11–13]. Phosphorylations of tuberous sclerosis complex 2 (TSC2) by AKT and ERK as well as phosphorylations of TSC1 by IKK β cause the suppression of TSC complex and the activation of mTOR signaling [14–16]. Although these three kinases share similar functions in regulating physiological activities, they are controlled by different stimuli. While AKT and ERK are regulated by growth factors and mitogens, IKK β is

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tightly controlled by pro-inflammatory cytokines, for example, TNF α and IL-1 β [17]. Upon TNF α stimulation, IKK β is activated and further triggers the degradation of I κ B α , mediating the expression of nuclear factor κ B (NF- κ B)-target genes. In addition to phosphorylate I κ B α , IKK β exerts its diverse functions by modulating several non-I κ B α -related proteins, such as FOXO3a [12], TSC1 [14], and ARD1 [18].

Recently, we and other groups demonstrated the critical role of IKK β in inflammation-associated cancer development [12,14,19,20]. Here, we continued the investigation and performed a detailed analysis of the underlying mechanisms by which TNF α induces HIF-1 α accumulation through IKK β . We found that the regulation of HIF-1 α by TNF α /IKK β occurred under both normoxic and hypoxic conditions, and was transcription independent. We provide further evidence showing that IKK β expression is positively correlated with VEGF expression in primary human breast cancer specimens. Our results demonstrate an important role for IKK β in inflammation-mediated HIF-1 α regulation.

Materials and methods

Constructs. The FLAG-IKK β and HA-HIF-1 α plasmids were generated as previously described [14,21].

Experimental reagents. We used antibodies to FLAG (F3165, Sigma–Aldrich, St. Louis, MO), HA (11666606001, Roche, Switzerland), IKK β (2684, Cell Signaling Technology, Danvers, MA; SC-7607, Santa Cruz Biotechnology, Santa Cruz, CA), HIF-1 α (GTX30105, GeneTex, San Antonio, TX), VEGF (Ab-3, Lab Vision, Fremont, CA), I κ B α (SC-371, Santa Cruz Biotechnology), and α -tubulin (T-5168, Sigma–Aldrich). TNF α (11 088 939 001) and CoCl₂ (C-3169) were purchased from Roche and Sigma–Aldrich, respectively.

siRNAs. MDA-MB-435 cells were transfected with IKK β SMART-pool siRNAs (M-003503, Dharmacon RNA Technologies, Lafayette, CO) or the ON-TARGETplus siCONTROL non-targeting siRNA pool (D-001206-13-05, Dharmacon RNA Technologies) using DharmaFECT Transfection Reagents (T-2001-02, Dharmacon RNA Technologies), and the cells were harvested for analysis 72 h after transfection.

Cell culture, stable transfectants and transient transfection. MCF-7 IKK β -stable transfectants were generated as previously described [14]. For transient transfection, cells at 50–60% confluence were transfected with DNA using stabilized non-viral (SN) liposomes (DNA:SN = 1 μ g:1 μ l) [22]. Six hours after transfection, the DNA-liposome mixture was removed and the fresh medium was added. Two days after transfection, the cells were harvested for analysis. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS).

Immunoblotting assays. Immunoblotting assays were performed as described previously [12]. Briefly, the cells were lysed in radioimmunoprecipitation assay-B (RIPA-B) buffer (1% Triton X-100, 150 mM NaCl, 20 mM Na₂PO₄, 1 mM PMSF, 3 μ g/ml aprotinin, 750 μ g/ml benzamidine, 2 mM Na₃VO₄, 5 mM NaF, pH 7.4). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sampling buffer was added to the cell lysates. After being boiled for 10 min, the protein samples were loaded onto an SDS–PAGE gel. For immunoblotting, proteins were subjected to SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes that had been pretreated with methanol. The membranes were blocked with 5% skim milk or 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer (10 mM Tris, 150 mM NaCl, pH 7.9) containing 0.05% Tween 20. The proteins were analyzed using specific antibodies as indicated. Horseradish peroxidase (HRP)-con-

jugated secondary antibodies and an enhanced chemiluminescence (ECL) kit were used for detection.

Real-time polymerase chain reaction (RT-PCR) and quantitative RT-PCR. First-strand cDNA was obtained from 1 μ g of total RNA isolated with the Trizol Reagent (15596-026, Invitrogen, Carlsbad, CA) using the SuperScript III First-Strand Synthesis System (18080-051, Invitrogen) and employing oligo(dT)₂₀ primers. For RT-PCR, the primers 5'-CACAGCCTGGATATGAA-3' and 5'-GAATTCCTGGTTATATATG-3' were used to amplify the HIF-1 α gene fragment. The PCR was performed in a total volume of 20 μ l Taq reaction buffer containing 6 nmol dNTPs, 20 pmol of each primer, 1 μ l DMSO and 0.2 μ l Taq polymerase. The PCR cycling conditions were as follows: one cycle at 94 °C for 5 min; 30 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min; and a final extension cycle at 72 °C for 10 min. For quantitative RT-PCR, the cDNAs were amplified in iQ SYBR Green Supermix (170-8880, Bio-Rad, Hercules, CA). The relative amount of mRNA was determined by performing RT-PCR in triplicate using specific primers with the following sequences: HIF-1 α forward, 5'-TCATCCAAGAAGCCCTAACG-3'; HIF-1 α reverse, 5'-TCGC TTTCTCTGAGCATTCTGC; β -actin forward, 5'-CGCCAAC CGCGAGAA GAT-3'; and β -actin reverse, 5'-CGTCACCGGAGTCCATCA-3'.

VEGF enzyme-linked immunosorbent assay (ELISA). The Quantikine Mouse VEGF ELISA Kit (MMV00, R&D, Minneapolis, MN) was used to measure the concentration of VEGF, and the assays were performed per the manufacturer's instructions. Briefly, 5×10^4 mouse embryonic fibroblast (MEF) cells were plated into each well of a 24-well plate, and the culture medium was harvested after 24 h. Assay Diluent RD1N and the cell culture samples were added into the ELISA plate and incubated for 2 h at room temperature. After the washing process, Mouse VEGF Conjugate was added and the samples were incubated for another 2 h. Substrate solution was then added and the optical density was measured after the reaction was terminated. The results are reported as the relative VEGF expression normalized to the total cell number at the time the culture medium was harvested.

Immunohistochemical (IHC) staining. IHC staining was performed as described elsewhere [12]. Briefly, primary human breast cancer specimens were incubated with antibodies to IKK β or VEGF, the presence of which was detected with biotin-conjugated secondary antibody and avidin-peroxidase and visualized with amino-ethylcarbazole chromogen. The staining intensity of the tumor samples was scored by taking the cross-product (H score) of the percentage of tumor samples at each level of staining intensity and ranked into four groups: negative, low, medium, and high, as described previously [23].

Statistical analyses. Statistical analyses were performed using either the Student's *t*-test or the Pearson chi-square test as indicated. A *P* value of <0.05 was considered statistically significant.

Results and discussion

TNF α induces HIF-1 α protein expression in multiple breast cancer cell lines

To study the effect of TNF α on HIF-1 α protein accumulation, we treated cells with 20 ng/ml of TNF α for 30, 60, or 180 min and harvested the cell lysates to measure the amount of HIF-1 α protein. Expression of HIF-1 α was increased more than threefold in MDA-MB-231 and MDA-MB-435 breast cancer cells following TNF α stimulation for 3 h under normoxic conditions (Fig. 1A). It is known that treatment of cells with transition metals such as CoCl₂ mimics hypoxic conditions, which stabilizes HIF-1 α protein in the cells. Thus, we performed similar experiments using cells that were first treated with CoCl₂ and found that TNF α consistently enhanced HIF-1 α expression in both MDA-MB-453 and HBL-100 cells

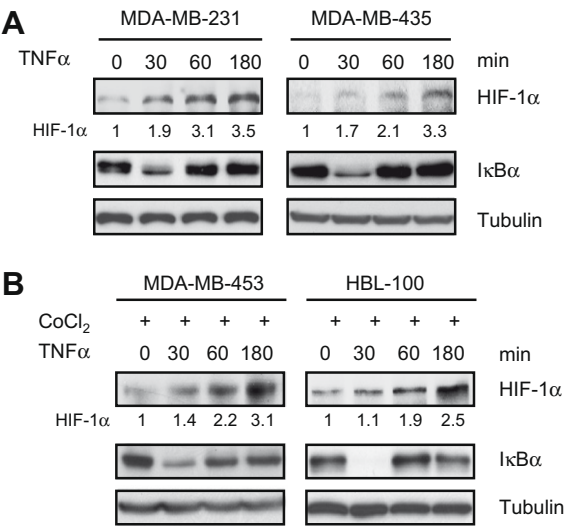


Fig. 1. TNFα induced HIF-1α protein accumulation in multiple breast cancer cell lines. (A) MDA-MB-231 and MDA-MB-435 cells were serum starved overnight and treated with 20 ng/ml TNFα, and samples were taken at the time points indicated. The expression level of HIF-1α was quantified with ImageJ software and normalized to the tubulin expression. (B) TNFα stimulation increased the protein level of HIF-1α in MDA-MB-453 and HBL-100 cells under hypoxia-mimicking conditions. Cells were treated as described in (A), except that they were preincubated with CoCl₂ (100 μM) for 6 h prior to analysis to mimic hypoxic conditions. The expression level of HIF-1α was quantified and normalized as described in (A).

(Fig. 1B). Taken together, these results suggest that TNFα induces HIF-1α expression under both normoxic and hypoxia-mimicking conditions.

Transcription of HIF-1α is not significantly affected by TNFα

We next wanted to determine the effects of TNFα stimulation on the HIF-1α mRNA level. As determined by RT-PCR and quantitative RT-PCR using β-actin as an internal control, the HIF-1α mRNA level in MDA-MB-231 and MDA-MB-435 cells did not change significantly in response to TNFα (Fig. 2A and B), suggesting that

the accumulation of HIF-1α protein is not caused by an increase in its transcription.

TNFα induction of HIF-1α protein is mediated by IKKβ

Based on our above findings that TNFα induces HIF-1α accumulation independent of its transcription, our next intention was to gain additional insight into the molecular mechanisms of HIF-1α induction by TNFα. Since IKKβ is the major kinase activated by TNFα stimulation, we next investigated whether IKKβ is required in this signaling pathway. We found an increased level of HIF-1α in IKKβ-stable transfectants that had been either treated with CoCl₂ or grown under hypoxic conditions (Fig. 3A). Transient transfection of wild-type (WT), but not kinase-dead (DN) IKKβ consistently enhanced HIF-1α expression (Fig. 3B). Depletion of IKKβ using siRNAs reduced the HIF-1α protein level (Fig. 3C), and pre-treatment of cells with the IKKβ inhibitor Bay 11-7082 completely blocked TNFα-induced HIF-1α accumulation (Fig. 3D), suggesting that IKKβ is required for the TNFα-induced enhancement of HIF-1α expression.

IKKβ expression is positively correlated with the VEGF protein level

It is now appreciated that VEGF is a direct target of HIF-1α and is involved in inflammation-mediated cancer development [24]. Therefore, we investigated the relationship between the expression of IKKβ and VEGF. We observed less VEGF expression in IKKβ knockout (IKKβ^{-/-}) MEFs than in wild-type MEFs (Fig. 4A). Consistent with these findings, immunohistochemical staining of 112 human primary breast tumor specimens from a previously described cohort of breast tumors [25] demonstrated that IKKβ expression was positively correlated with VEGF level ($P < 0.05$; Fig. 4B and C). Taken together, these results further strengthen the notion of HIF-1α upregulation by IKKβ and the physiological importance of IKKβ in inflammation-mediated VEGF expression.

TNFα has been shown to regulate HIF-1α at the transcriptional and translational levels as well as by transactivation. Here, we have demonstrated that TNFα causes the accumulation of HIF-1α protein, but not its mRNA through IKKβ. Early work suggested that TNFα/IKKβ may control protein translation through the mamma-

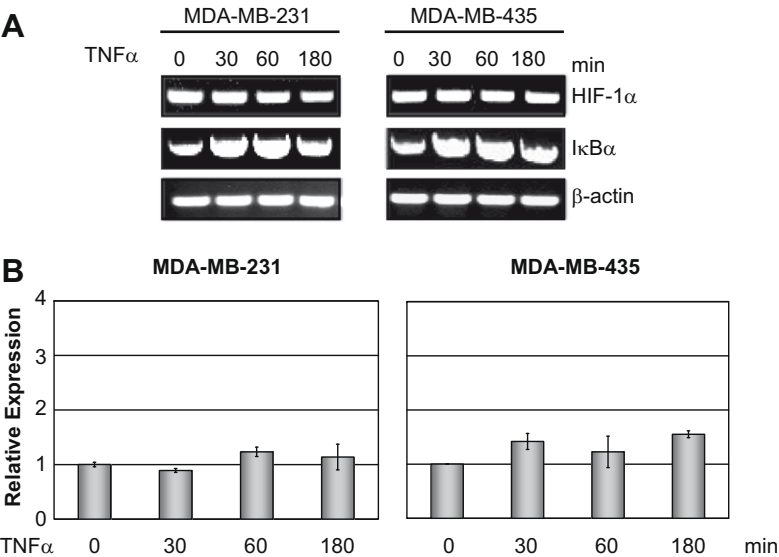


Fig. 2. TNFα stimulation does not change the level of HIF-1α mRNA (A) MDA-MB-231 and MDA-MB-435 cells were serum starved overnight and treated with 20 ng/ml TNFα, and samples were taken at the time points indicated. HIF-1α mRNA expression was measured by RT-PCR analysis. (B) RNA was isolated and subjected to quantitative RT-PCR. The HIF-1α mRNA level before TNFα treatment (TNFα 0 min) was arbitrarily set to 1 U following normalization to the level of β-actin mRNA. The results are representative of three independent experiments.

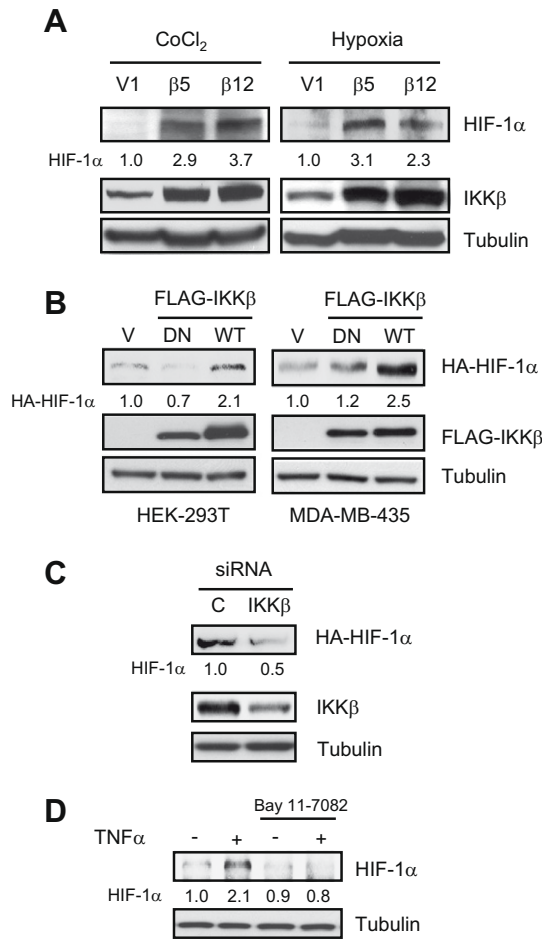


Fig. 3. IKKβ is required for TNFα-induced HIF-1α expression. (A) Enhanced HIF-1α expression was observed in the independent IKKβ stable clones β5 and β12 which are generated in MCF-7 cells. The cells were either preincubated with CoCl₂ (100 μM) or subjected to hypoxic conditions (1% O₂) 6 h before analysis. The expression level of HIF-1α was quantified with ImageJ software and normalized to the tubulin expression. (B) Wild-type, but not kinase-dead IKKβ, enhanced HIF-1α expression. Control vector and various FLAG-IKKβ constructs were cotransfected with HA-HIF-1α into HEK-293T or MDA-MB-435 cells, and HA-HIF-1α expression was detected by immunoblotting 2 days after transfection. The expression level of HIF-1α was quantified with ImageJ software and normalized to the tubulin expression. V, vector control; DN, kinase-dead FLAG-IKKβ; WT, wild-type FLAG-IKKβ. (C) Depletion of IKKβ reduced the expression of HIF-1α. IKKβ or control siRNAs were cotransfected with HA-HIF-1α into MDA-MB-435 cells and the cells were harvested for analysis 3 days later. The protein level of HA-HIF-1α was normalized to the tubulin expression. (D) The IKKβ inhibitor Bay 11-7082 (40 μM) blocked TNFα-induced HIF-1α accumulation. MDA-MB-435 cells were pretreated with Bay 11-7082 for 45 min and then stimulated with TNFα for another 3 h. The expression level of HIF-1α was normalized to the tubulin level.

lian target of rapamycin (mTOR) signaling pathway [14]. Further investigation will be necessary to determine whether the IKKβ-induced HIF-1α accumulation is mediated by translational or post-translational regulation.

Three oncogenic kinases, AKT, ERK, and IKKβ, are frequently activated in many human cancers. In addition to targeting the same tumor suppressors, these kinases can also enhance the expression or activity of common oncoproteins [12–14], including HIF-1α, to promote tumor progression. Therefore, targeting all three of these kinases with inhibitors is an attractive strategy for cancer therapy.

In summary, we have shown that IKKβ, the major downstream kinase of TNFα, is essential for TNFα-mediated HIF-1α regulation, which further enhances VEGF expression (Fig. 4D). Our study provides new molecular explanations for understanding TNFα-in-

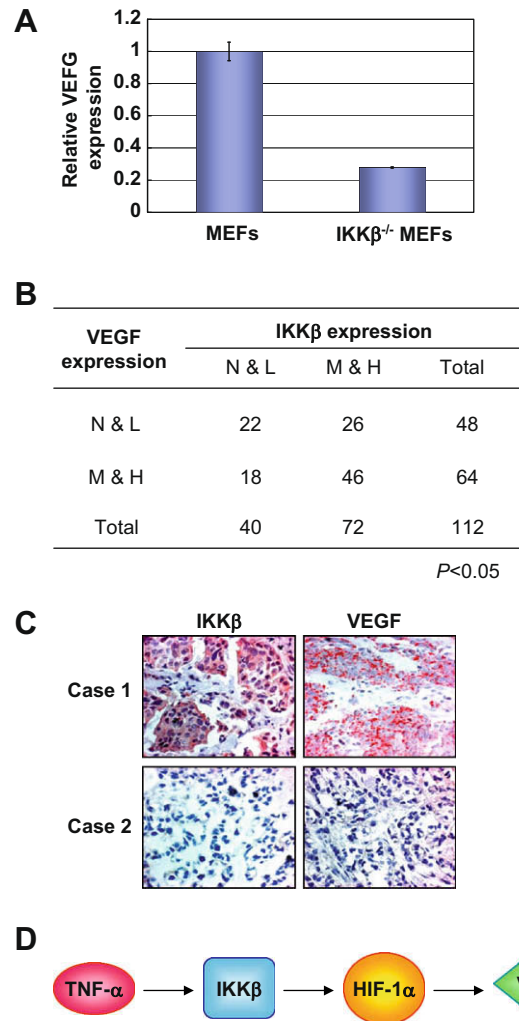


Fig. 4. IKKβ expression was correlated with VEGF protein level. (A) IKKβ knockout (IKKβ^{-/-}) MEFs had less VEGF expression than wild-type MEFs (*P* < 0.05). The level of VEGF in the culture medium was determined by ELISA and normalized to the cell number. Statistical significance was calculated using the Student's *t*-test. (B) IKKβ expression was positively correlated with the VEGF level in 112 primary human breast cancer specimens. Correlation analyses were performed using the Pearson chi-square test (*P* < 0.005). N, negative; L, low; M, medium; H, high. (C) Two representative specimens with consecutive tissue sections. (D) A model showing that TNFα induces HIF-1α accumulation through IKKβ, which increases VEGF expression.

duced HIF-1α protein accumulation, and points to IKKβ as a critical signaling component in facilitating the response to TNFα. These observations may advance our knowledge of the molecular mechanisms of inflammation-mediated angiogenesis and cancer development.

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