Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor κB activation

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Tumour necrosis factor α (TNF-α) binds to its receptor (TNFR1) and activates both death- and inflammation/survival-related signalling pathways. The inflammation and survival-related signalling cascade results in the activation of the transcription factor, nuclear factor κB (NF-κB) and requires recruitment of receptor-interacting protein (RIP) to TNFR1. The indispensable role of RIP in TNF-induced NF-κB activation has been demonstrated in $RIP^{-/-}$ mice and in cell lines derived from such mice. In the present study, we show that the TNF-α-induced accumulation of hypoxia-inducible factor 1α (HIF-1α) protein in normoxic cells is RIP-dependent. Exposing fibroblasts derived from $RIP^{-/-}$ mice to either cobalt or PMA resulted in an equivalent HIF-1α induction to that seen in $RIP^{+/+}$ fibroblasts. In contrast, $RIP^{-/-}$ cells were unable to induce HIF-1α in response to TNF-α. Further, transient transfection of NIH 3T3 cells with an NF-κB super-repressor plasmid (an inhibitor of NF-κB activation) also prevented HIF-1α induction by TNF-α. Surprisingly, although HIF-1α mRNA levels remained unchanged after induction by TNF, induction of HIF-1α protein by the cytokine was completely blocked by pretreatment with the transcription inhibitors actinomycin D and 5,6-dichlorobenzimidazole riboside. Finally, TNF failed to induce both HIF-1α, made resistant to von Hippel–Lindau (VHL), and wild-type HIF-1α transfected into $VHL^{-/-}$ cells. These results indicate that HIF-1α induction by TNF-α in normoxic cells is mediated by protein stabilization but is nonetheless uniquely dependent on NF-κB-driven transcription. Thus the results describe a novel mechanism of HIF-1α up-regulation and they identify HIF-1α as a unique component of the NF-κB-mediated inflammatory/survival response.

Key words: hypoxia-inducible factor 1α, nuclear factor κβ, receptor-interacting protein, tumour necrosis factor.

INTRODUCTION

Hypoxia-inducible factor (HIF)-1 is a heterodimeric transcription factor composed of the basic helix–loop–helix PAS (Per-Arnt-Sim)-domain-containing proteins HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT, HIF-1β) [1]. HIF-1α and HIF-1β mRNAs are constitutively expressed in a variety of mammalian cell lines under normoxic and hypoxic conditions [2]. At the protein level, HIF-1α is markedly elevated by hypoxia, whereas HIF-1β expression remains relatively constant regardless of oxygen tension [3]. Under normoxia, HIF-1α is normally unstable and is degraded via the ubiquitin–proteasome pathway. Under hypoxia, HIF-1α is resistant to proteasomal degradation, and the stabilized protein heterodimerizes with HIF-1β, resulting in DNA binding and transactivation of HIF-responsive genes [4–6]. In addition to hypoxia, other inducers of HIF-1α expression in normoxic cells include somatic mutations that either activate oncogene products (e.g. Src, Ras) or inactivate tumour supressor proteins [von Hippel–Lindau (VHL), p53 and PTEN], bivalent metals, PMA, iron chelators, growth factors and reactive oxygen species [6–14].

HIF-1 activates the transcription of genes encoding proteins that affect metabolic adaptation (glucose transporters and glycolytic enzymes), oxygen delivery (erythropoietin), angiogenesis (vascular endothelial growth factor) and cell survival (insulin-like growth factor 1) [15]. There is growing evidence that HIF-1 is involved in the inflammatory process by regulating angiogenesis [16,16a] and development of immune cells [17,18]. Further, HIF-1-regulated gene products are likely to play essential roles in tumour progression and/or survival *in vivo* [19–23].

Tumour necrosis factor (TNF)-α, a pro-inflammatory cytokine, is an endogenous mediator of inflammation, septic shock, anti-viral responses and apoptotic cell death [24]. TNF-α elicits its complex biological repertoire through the individual or co-operative action of two TNF receptors of molecular mass 55 kDa (TNFR1) and 75 kDa (TNFR2) respectively, which are expressed in most cell types [25]. TNFR1 signalling leads to cytotoxic and inflammatory/survival responses [26]. TNF-induced inflammatory/survival signalling requires activation of transcription factors, nuclear factor κB (NF-κB) and activator protein 1 (AP-1) [27]. Receptor-interacting protein (RIP) is a serine/threonine protein kinase that interacts with Fas/apoptosis-1 (CD95) and TNFR1 [28]. Gene knockout experiments suggest that RIP is required for TNFR1-mediated NF-κB activation, but is not required for TNFR1-mediated apoptosis [29–32].

Under normal conditions, NF-κB is present within the cytoplasm in an inactive state, bound to inhibitory κB (IκB) protein [33]. Stimulation with TNF-α initiates an intracellular signalling cascade that results in the phosphorylation of IκB by IκB kinase. Phosphorylation and subsequent ubiquitination of IκB targets the protein for degradation by the 26 S proteasome [34,35]. The degradation of IκB leads to the release of NF-κB, allowing it to translocate to the nucleus, where it orchestrates the co-ordinated

Abbreviations used: AP-1, activator protein 1; HA, haemagglutinin; HIF, hypoxia-inducible factor; IκB, inhibitory κB; MEF, mouse embryo fibroblasts; NF-κB, nuclear factor κB; RIP, receptor-interacting protein; RT, reverse transcriptase; TNF, tumour necrosis factor; TNFR, TNF receptor; VHL, von Hippel–Lindau.

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transcription of a number of pro-inflammatory genes, including cytokines, chemokines and cell adhesion molecules [36].

Recently, the relationship between inflammation and carcinogenesis has become apparent [37], and NF-κB has become an exciting novel target for cancer drug development [38]. Several studies have reported the ability of TNF-α to promote HIF-1α accumulation in fibroblasts, epithelial cells and primary inflammatory polymorphonuclear leucocytes, although the molecular pathways that mediate this response are not well understood [39–42]. The involvement of NF-κB in both inflammation and cancer prompted us to examine whether TNF induction of HIF-1α proceeds via an NF-κB-dependent pathway. In the present study, we show that accumulation of HIF-1α in response to TNF-α is indeed mediated by the TNFRI-RIP-NF-κB signalling cascade. Unlike the case with other inducers of HIF-1α, including hypoxia, induction of the transcription factor by TNF-α is transcription-dependent.

MATERIALS AND METHODS

Reagents

TNF-α (recombinant human TNF-α) was obtained from R&D Systems. Actinomycin D, 5,6-dichlorobenzimidazole riboside, doxycycline and cobalt chloride were purchased from Sigma. PMA was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other materials were obtained in the highest available grade.

Cells and transient transfection

MCF-7, HEK-293, NIH 3T3 cells (obtained from A.T.C.C.), Rat1 cells stably transfected with a doxycycline-inducible inhibitor of AP-1 (dominant-negative c-Jun plasmid, a gift from Dr M. Birrer, NCI, MD, U.S.A.), wild-type mouse embryo fibroblasts (MEF) and MEF derived from RIP$^{-/-}$ mice [43] were cultured in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD, U.S.A.). A549 cells (obtained from A.T.C.C.) were grown in F12-K medium (Gibco BRL, Gaithersburg, MD, U.S.A.). Cell viability was greater than 90 % in each experimental condition, as determined by Trypan Blue staining.

For transient transfection of NF-κB super-repressor plasmid [29], (9–11) $\times$ 10^5 cells were plated in 6 cm dishes and transfected the following day in the presence of FuGene 6 (Roche Molecular Biochemicals, Pleasanton, CA, U.S.A.). After 24 h, cells were exposed to TNF for 2 h, lysed, and HIF-1α levels were determined by Western blotting. For transient transfection of reporter plasmids, (8–9) $\times$ 10^4 cells were plated in 6-well plates. The next day, 10 ng cytomegalovirus promoter-driven *Renilla* luciferase plasmid (Promega) was cotransfected with luciferase reporter plasmids under the transcriptional control of either NF-κB (a gift from Dr M. Birrer) or HIF-1α response elements [44] (a gift from Dr G. Melillo, NCI, MD, U.S.A.).

For transient transfection of wild-type and point-mutated HIF-1α, a cytomegalovirus driven haemagglutinin (HA)-tagged HIF-1α construct was originally obtained from Dr D. Livingston (Dana-Farber Cancer Institute, Boston, MA, U.S.A.). Using primers containing sites for *Bam*HI (5′) and *Not*I (3′), this insert was PCR-amplified, subcloned into PCDNA3.1 (Invitrogen, Carlsbad, CA, U.S.A.) and confirmed by sequencing. This latter construct was used for all wild-type HIF-1α transfections and as a template for subsequent mutations. To make site-specific mutations, complementary primers containing desired point mutations were constructed and PCR amplification was performed in accordance with XL site-directed mutagenesis kit obtained from Promega. For transfection, cells were seeded at 60 % confluency, 24 h before transfection and 3 μg plasmid DNA was used per 10 cm dish. Transfections were performed using Fugene (Roche, Indianapolis, IN, U.S.A.), according to the manufacturer's instructions.

Luciferase assay

Six hours after transfection with luciferase reporter plasmids, cells were treated with drugs. NF-κB-dependent luciferase activity was measured 6 h later and HIF-1α-dependent luciferase activity was measured 10 h later, using the Dual-Luciferase Reporter Assay System (Promega). Results were corrected for the activity of *Renilla* luciferase.

Western blotting

Cells were lysed and nuclear extracts were prepared as described previously [45]. HIF-1α protein in human cells was detected in 20 μg of nuclear extract using monoclonal HIF-1α antibody (1:500; Transduction Laboratories, Lexington, KY, U.S.A.) and HIF-1α in non-human cells was detected in 30 μg of nuclear extract using monoclonal HIF-1α antibody (1:750; Novus, Littleton, CO, U.S.A.).

Quantitative reverse transcriptase (RT)–PCR analysis for HIF-1α expression

The real-time quantification of HIF-1α mRNA was performed using SYBR Green I dye (Applied Biosystems, Foster City, CA, U.S.A.) with the following primer pairs. Mouse HIF-1α forward: 5′-CAAGTCAGCAACGTGGAAGGT-3′; mouse HIF-1α reverse: 5′-CTGAGGTTGGTTACTGTTGGTATCA-3′; human HIF-1α forward: 5′-TCCAGTTACGTTCCTTCGATCA-3′; human HIF-1α reverse: 5′-TTTGAGGACTTGCGCTTTCA-3′.

SYBR Green I, double-stranded DNA-binding dye, was detected using the laser-based ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR amplification was performed using an optical 96-well reaction plate and caps. The final reaction mixture of 25 μl consisted of 200 nM of each primer, 1 $\times$ SYBR Green PCR Master Mix (Applied Biosystems), containing a reference dye and cDNA under the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The cDNAs were prepared from each RNA sample using a TaqMan Reverse Transcription Kit (Applied Biosystems).

RESULTS

TNF-α induces HIF-1α protein in a time- and transcription-dependent manner in NIH 3T3 cells

NIH 3T3 cells were treated with 10 or 20 ng/ml TNF-α for 2, 4, or 6 h, or with cobalt chloride (100 μM) for 3 h. HIF-1α protein was then measured by Western-blot analysis (Figure 1A). Both concentrations of TNF-α induced the accumulation of HIF-1α protein to a similar degree and in both cases the effect was transient, with HIF-1α levels returning to control level by 6 h. The effect of cobalt chloride, which persists for many hours (results not shown), is shown as a positive control. Induction of HIF-1α protein by cobalt chloride is transcription-independent. Thus pre-treatment of NIH 3T3 cells with actinomycin D (10 μM for 20 min) had no effect on the ability of cobalt to induce HIF-1α protein (Figure 1B). In contrast, accumulation of HIF-1α protein in response to TNF-α was completely prevented by pretreatment with either actinomycin D or a second transcription inhibitor, 5, 6-dichlorobenzimidizole riboside (70 μM for 20 min;

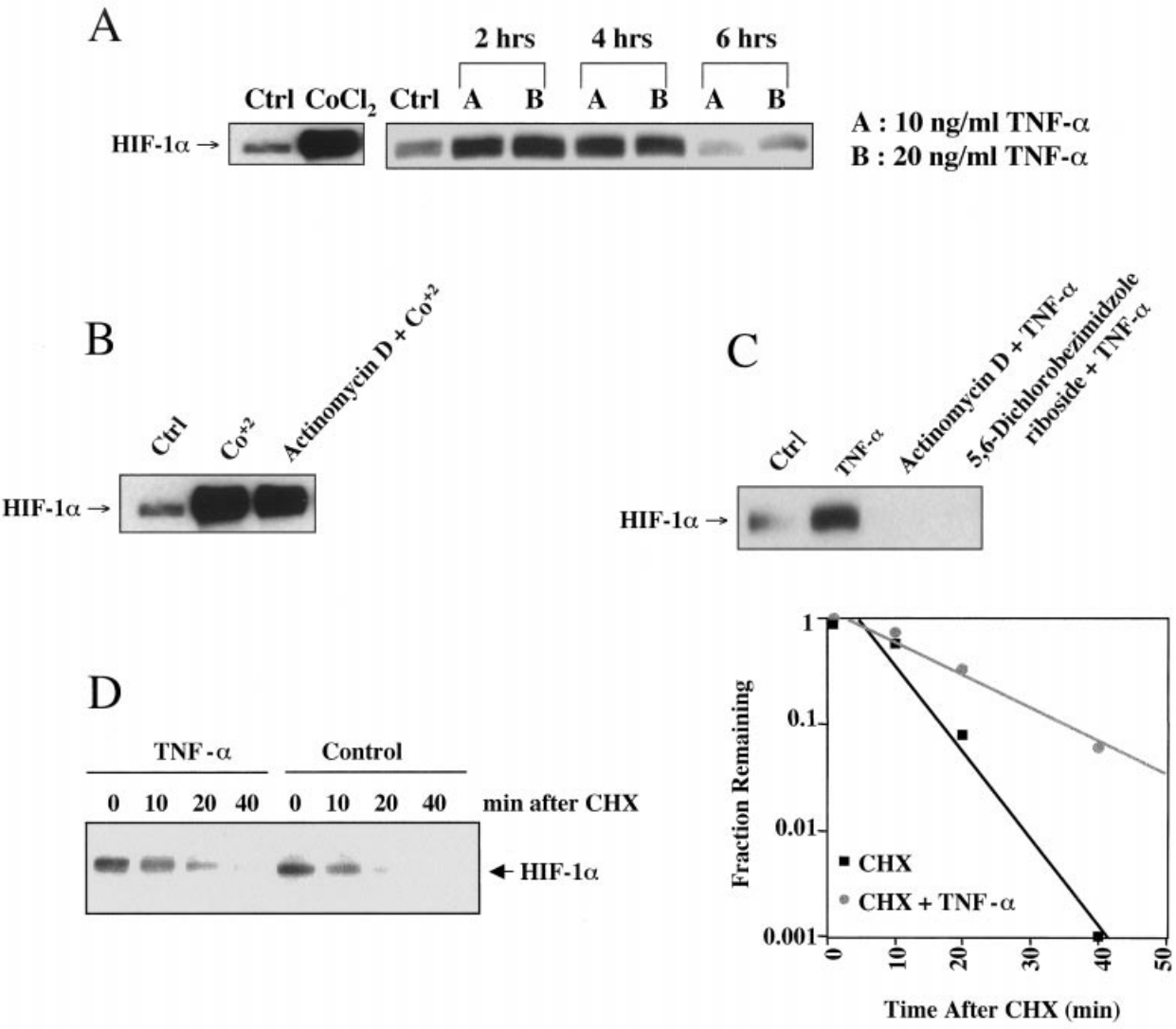

Figure 1 TNF-α induction of HIF-1α protein in NIH 3T3 cells is time-dependent

(**A**) Cells were treated with cobalt chloride (100 μM) for 3 h, or TNF-α (10 and 20 ng/ml) for 2, 4 or 6 h. HIF-1α protein was assayed as described in the Materials and methods section. (**B**) Cells were treated with cobalt as in (**A**) but in the presence of actinomycin D, and HIF-1α protein was monitored by Western-blot analysis. (**C**) Cells were treated with TNF-α (10 ng/ml) alone or in the presence of either actinomycin D or 5,6-dichlorobenzimidazole riboside and HIF-1α protein was monitored by Western blotting. (**D**) Cycloheximide (100 μg/ml) was added to cells either previously untreated or exposed to TNF-α. At the times shown, cells were lysed and HIF-1α protein was detected by Western blot. The film was scanned and band densities were determined by densitometry using NIH Image software, and the results graphically displayed using Cricket Graph software. TNF-α treatment increased the half-life of HIF-1α protein by 1.7-fold.

Figure 1C). However, as determined by RT–PCR, HIF-1α mRNA levels in 3T3 cells did not change significantly in response to cytokine (results not shown). Thus although transcription is required in the pathway utilized by TNF-α to induce HIF-1α protein, transcription of HIF-1α itself is not significantly affected by TNF-α.

TNF induction of HIF-1α protein in mouse fibroblasts requires RIP

Because HIF-1α mRNA levels were not affected by TNF-α, we reasoned that the transcription dependence of TNF induction of HIF-1α lay upstream of HIF. Therefore, we next investigated the requirement for RIP in this signalling pathway. Wild-type MEF and MEF derived from RIP$^{-/-}$ mice were treated with TNF-α for 2 h, or PMA or cobalt for 4 h, and HIF-1α protein levels were examined by Western-blot analysis (Figure 2A). PMA was chosen because it activates NF-κB and induces HIF-1α protein in a RIP-independent fashion, whereas cobalt stabilizes HIF-1α protein without induction of NF-κB [13,46,47]. Although both PMA and cobalt induced HIF-α protein to an equivalent degree in both wild-type and RIP$^{-/-}$ cells, TNF-α failed to induce HIF-1α accumulation in the RIP$^{-/-}$ cells. Similarly, while all three agents stimulated HIF-dependent transcriptional activity in wild-type MEF, TNF-α was unable to do so in RIP$^{-/-}$ MEF (Figure 2B).

TNF induction of HIF-1α protein is mediated by NF-κB

Since RIP is necessary for TNF induction of NF-κB, the requirement for RIP in TNF-dependent HIF-1α induction prompted us to examine whether NF-κB mediated the response

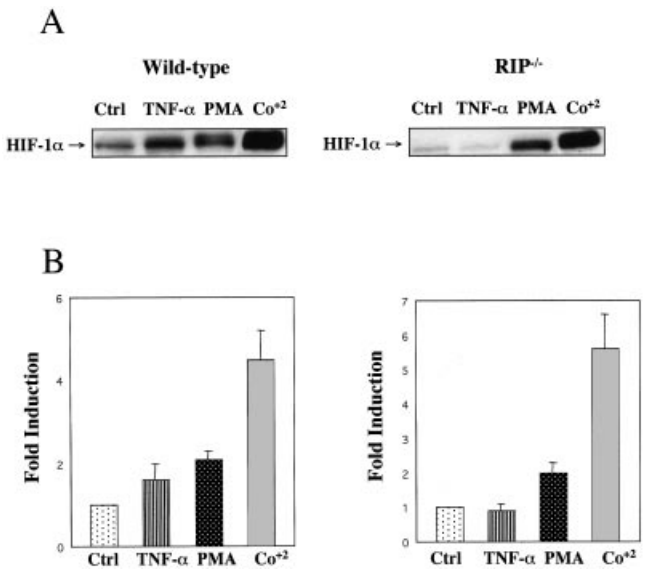

Figure 2 TNF induction of HIF-1α protein requires RIP

(**A**) Wild-type and RIP$^{-/-}$ MEF were treated for 2 h with TNF (10 ng/ml), or for 4 h with either PMA (1 μM) or cobalt chloride (100 μM) and HIF-1α protein was monitored by Western blot experiments. (**B**) MEF were transiently co-transfected with an inducible nitric oxide synthase promoter-containing HIF-dependent luciferase reporter plasmid and a *Renilla* luciferase plasmid. After 6 h cells were exposed to drugs as shown and HIF-dependent reporter activity, corrected for *Renilla* activity, was determined 10 h later. Results for wild-type cells are shown in the left panel and for RIP$^{-/-}$ in the right panel. Results reflect the means $\pm$ S.E. for three independent experiments.

to TNF-α. First, to confirm the absence of TNF-inducible NF-κB activity in RIP$^{-/-}$ MEF, we transfected wild-type and RIP$^{-/-}$ MEF with an NF-κB luciferase reporter plasmid. The next day, cells were challenged with either TNF-α or PMA, and NF-κB-dependent luciferase activity was monitored 6 h later. For wild-type MEF, both TNF-α and PMA significantly stimulated NF-κB-dependent transcription, whereas in RIP$^{-/-}$ cells only PMA was able to do so (Figure 3A). These results confirm the RIP dependence of TNF induced NF-κB activity in fibroblasts. Furthermore, they show that the ability of PMA to induce NF-κB activity is independent of RIP.

Next, we transfected NIH 3T3 cells with an NF-κB super-repressor plasmid (IκB mutated to resist proteasome-mediated degradation) and, 24 h later, challenged these cells with TNF-α. Cells were lysed 2 h after TNF exposure and HIF-1α protein was measured by Western-blot analysis. Transfection with NF-κB super-repressor caused a dose-dependent reduction in the level of HIF-1α obtained in response to TNF-α (Figure 3B). Because TNF-α also induces AP-1 activity, we examined whether AP-1 inhibition could interfere with TNF-dependent accumulation of HIF-1α. In contrast with the data obtained using the NF-κB super-repressor plasmid, we found that TNF-treated Rat1 cells stably expressing dominant-negative c-Jun were able to induce HIF-1α protein to a similar degree whether or not AP-1 was inhibited (Figure 3C). These results thus specify the TNF-RIP-NF-κB transcriptional pathway as necessary and sufficient to promote HIF-1α protein accumulation in normoxia.

TNF-α induces HIF-1α protein in several epithelial cell lines as well as fibroblasts

Because the previous experiments were all performed using fibroblasts, we wished to determine whether epithelial cell lines behaved in a similar fashion. Thus three epithelial cell lines, the human embryonal kidney cell line HEK-293, the lung carcinoma cell line A549 and the breast carcinoma cell line MCF7 were exposed to TNF-α for 4 h and then examined for HIF-1α protein expression by Western blotting. In each case, HIF-1α protein was strongly expressed (Figure 4A). In A549 cells, RT–PCR analysis revealed that, as in fibroblasts, TNF-dependent HIF-1α

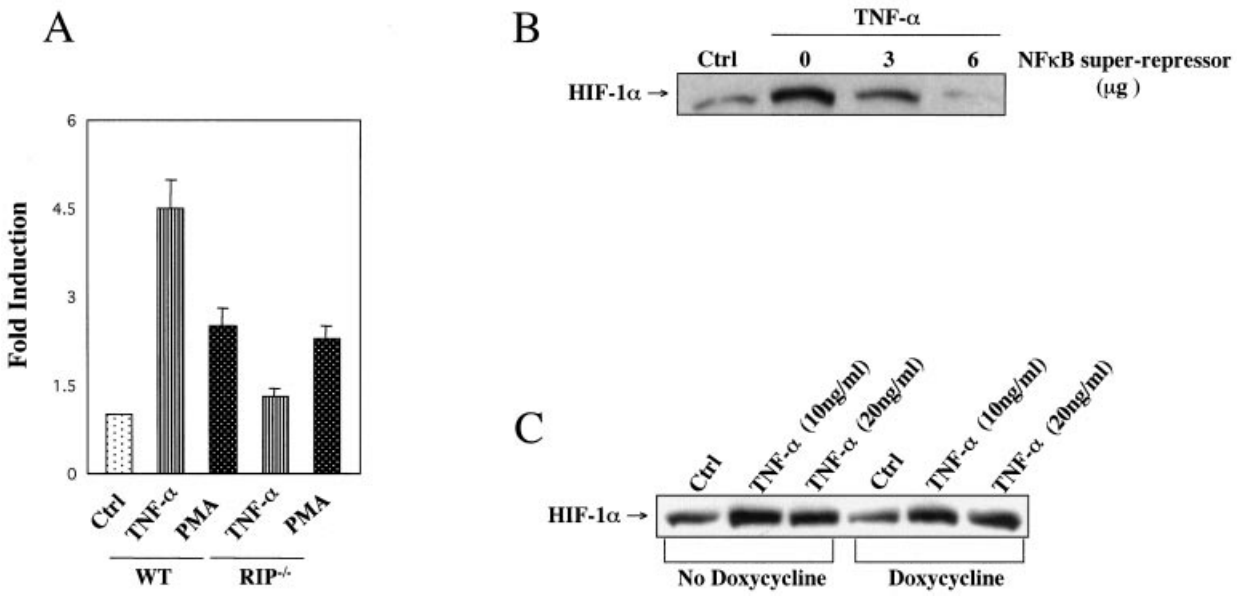

Figure 3 TNF induction of HIF-1α protein requires NF-κB

(**A**) Wild-type and RIP$^{-/-}$ MEF were transiently co-transfected with an NF-κB reporter plasmid and a *Renilla* luciferase plasmid. Drugs were added and NF-κB-dependent reporter activity, normalized for *Renilla* activity, was assayed after an additional 6 h. Results are shown as means $\pm$ S.E. for three independent experiments. (**B**) NIH 3T3 cells were transiently transfected with an NF-κB super-repressor plasmid and exposed to TNF-α (10 ng/ml) 24 h later. HIF-1α protein was monitored after 2 h by Western blot studies. (**C**) Rat-1 cells, untreated or treated with doxycycline (1 μg/ml, 4 h) to induce AP-1 inhibition, were challenged with TNF-α for 2 h, and HIF-1α protein was detected by Western-blot analysis.

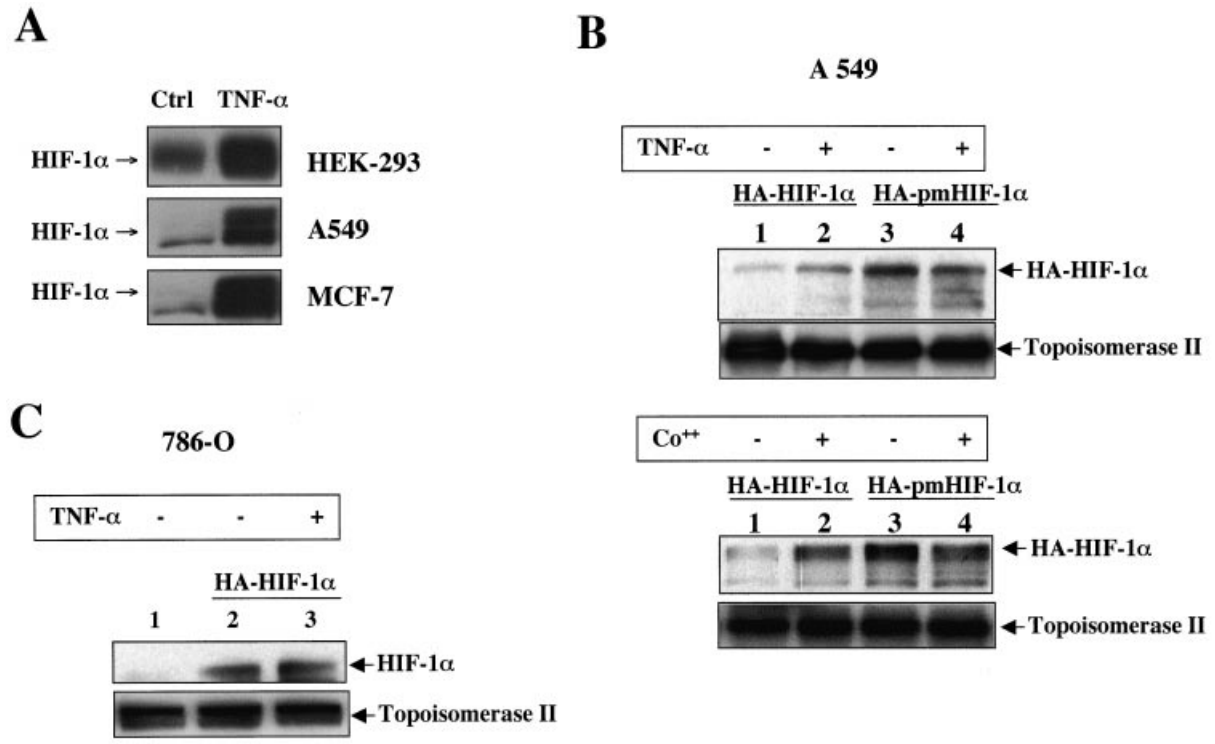

Figure 4 TNF induces HIF-1α protein in multiple epithelial cell lines

(**A**) Cells were treated with TNF-α (10 ng/ml) for 4 h and HIF-1α protein was monitored by Western-blot analysis. (**B**) A549 cells were transiently transfected with either wild-type HA-HIF-1α or HA-HIF-1α whose Pro^{402} and Pro^{564} residues were changed to alanine (HA-pmHIF-1α). Twenty-four hours after transfection, cells were challenged with either cobalt chloride or TNF-α for 4 h, and the level of transfected HIF-1α protein was detected by blotting nuclear extract for the HA epitope tag. Blots were also probed for topoisomerase II to demonstrate equal loading of nuclear protein in each lane. (**C**) 786-O cells, lacking both endogenous HIF-1α and VHL, were transfected with wild-type HA-HIF-1α and challenged with TNF 24 h later. Levels of transfected HIF-1α protein were monitored 4 h after TNF by Western blotting of nuclear extracts. Blots were probed for topoisomerase II to demonstrate equal loading of nuclear protein in each lane.

protein accumulation was not accompanied by any change in HIF-1α mRNA level (results not shown).

TNF-α fails to induce HIF-1α protein that is insensitive to VHL

TNF induction of HIF-1α, although transcription-dependent, was not accompanied by an increase in HIF-1α mRNA level. Therefore we investigated the possible involvement of VHL, the HIF-ubiquitinating E3 ligase, in this response. A549 cells were transfected with either wild-type HA-tagged HIF-1α or proline-mutated HA-tagged HIF-1α. Simultaneous mutation of HIF-1α at Pro^{402} and Pro^{564} prevents VHL binding [48–51], thus stabilizing HIF-1α in normoxic cells. The data in Figure 4(B) shows that while transiently transfected wild-type HA-HIF-1α can be induced to accumulate further by TNF, proline mutated HA-HIF-1α cannot. Lastly, we transiently transfected 786-O cells, which lack both endogenous HIF-1α and VHL [52], with wild-type HA-HIF-1α and we challenged these cells with TNF. As shown in Figure 4(C), TNF failed to promote the induction of wild-type HIF-1α in these cells.

DISCUSSION

Inflammatory cytokines, including TNF-α, have been described previously to induce HIF-1 DNA-binding concomitant with an increase in either HIF-1α mRNA or protein, although the published results are somewhat contradictory. Thus Thornton et al. [53] have reported that brief exposure of gingival and synovial fibroblasts to TNF-α resulted in elevated HIF-1α mRNA level and DNA-binding activity, whereas Hellwig-Burgel et al. [39] reported no change in HIF-1α mRNA or protein level after treatment of HepG2 cells by TNF. Nevertheless, these investigators confirmed that TNF-α induced HIF DNA binding. Using a wound model, Albina et al. [40] reported that TNF-α did not affect HIF-1α mRNA in cultures of primary inflammatory cells, although the cytokine did cause an increase in HIF-1α protein level. In contrast, neutrophils recovered from the wound site were rich in TNF-α and contained elevated levels of HIF-1α mRNA [40]. Although these authors found no role for O_2 radicals in this process, Haddad and Land [41], using an alveolar epithelium model, have reported that TNF-dependent induction of HIF DNA binding and HIF-1α protein was reactive oxygen species-sensitive. In contrast, using a renal tubular epithelium model, Sandau et al. [42] found that O_2 radicals inhibited both TNF-mediated accumulation of HIF-1α protein and HIF-dependent transcriptional activity. Although these conflicting results may be reflective of the different cell types and treatment conditions explored by these investigators, it is clear that TNF is able to enhance HIF activity in fibroblasts, neutrophils and epithelial cells.

Since several of the earlier studies have suggested that TNF-induced activation of HIF is part of the inflammatory response triggered by the cytokine, we have examined whether TNF-induced activation of the pro-inflammatory transcription factor NF-κB may play a role in HIF-1α induction. Using NIH 3T3 cells, we ascertained that two inhibitors of transcription, actino-

mycin D and 5,6-dichlorobenzimidazole riboside, completely blocked the transient accumulation of HIF-1α protein in response to TNF-α. This is in contrast with the lack of transcription dependence of cobalt, a chemical mimic of hypoxia, which post-translationally stabilizes HIF-1α protein. Nonetheless, and in agreement with several previous studies mentioned above, TNF-α did not affect HIF-1α mRNA level. The most conservative explanation of these results is that TNF-α induces transcription of a labile protein capable of stabilizing HIF-1α. Thus TNF-dependent HIF induction proceeds via a previously unrecognized pathway.

Although the enhancement of HIF-dependent transcriptional activity produced by TNF-α and PMA is small, these values are in agreement with previous reports [13,42]. However, while HIF inducers, such as cobalt and PMA, functioned equally well in the presence and absence of RIP expression, TNF-α absolutely required RIP to promote HIF-1α protein accumulation and transcriptional activity. Similarly, while PMA induced equivalent amounts of NF-κB-dependent transcriptional activity in wild-type and RIP$^{-/-}$ MEF, TNF induction of NF-κB activity was reduced by nearly 95% in RIP$^{-/-}$ cells. PMA was used in the present study because it is known to stimulate NF-κB via a novel IκB kinase complex, whose activation is neither utilized nor required by TNF [47]. Thus our results for PMA demonstrate the functional integrity of the NF-κB—HIF pathway in the RIP$^{-/-}$ cells.

Finally, transient transfection of an NF-κB super-repressor (proteasome-resistant mutated IκB) dose-dependently inhibited HIF-1α induction by TNF-α, whereas inhibition of AP-1 had no effect. Taken together, these results support a key role for the RIP-NF-κB signalling pathway in mediating the effects of TNF-α on HIF-1α, and they suggest that the HIF-1α-stabilizing protein induced by TNF-α is an NF-κB transcriptional target. This response to TNF is not limited to fibroblasts, since a renal, breast and lung epithelial cell line each responds similarly. The transience of TNF-α-induced HIF-1α protein is consistent with the transient kinetics of TNF-α-induced NF-κB signalling [54].

The identity of this stabilizing protein remains unknown but its probable method of action may be interference in VHL-mediated HIF-1α ubiquitination. This hypothesis is supported by our finding that TNF cannot further increase the cellular level of HIF-1α protein mutated at Pro^{402} and Pro^{564} residues, as this HIF mutant is fully resistant to VHL-mediated HIF-1α ubiquitination [55]. Furthermore, TNF failed to increase HIF-1α levels in 786-O cells that lack endogenous VHL [52]. Taken together, these results suggest that the factor responsible for TNF-mediated HIF-1α induction in some way interferes with the ability of VHL to interact with HIF-1α. We are currently investigating whether TNF promotes inhibition of the proline hydroxylase responsible for modifying HIF-1α.

Since the TNF-RIP-NF-κB signalling pathway transduces inflammatory/survival signals, TNF-induced HIF-1α may participate in this process. In fact, this is in agreement with the known physiological role of the protein. Thus HIF-1α transactivates genes encoding inducible nitric oxide synthase, haem oxygenase-1, erythropoietin and vascular endothelial growth factor, all of which may be involved in the inflammatory process [56–59]. Since chronic inflammation provides a physiological environment that favours DNA damage and genetic alterations [37], the link between inflammation and cancer is clear. One of the key transcriptional components common to both processes is NF-κB. Although NF-κB is induced by inflammatory cytokines such as TNF-α, its activity is constitutively elevated in many different types of cancer [38]. HIF-1α is recognized as an important survival gene for cancer cells and is frequently found to be expressed in normoxic as well as hypoxic tumours [21,22]. Our results suggest that NF-κB promotes HIF-1α expression in normoxic cancer cells via transcriptional induction of an HIF-1α-stabilizing protein. By demonstrating a direct link between NF-κB and HIF-1α, our results provide further evidence regarding the role of NF-κB in carcinogenesis.

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