A non-hypoxic, ROS-sensitive pathway mediates TNF-α-dependent regulation of HIF-1α

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Abstract A non-hypoxic, reactive oxygen species (ROS)-sensitive pathway mediating tumor necrosis factor-α (TNF-α)-dependent regulation of hypoxia-inducible factor-1α (HIF-α) was investigated in vitro. TNF-α mediated the translocation of HIF-1α, associated with up-regulating its activity under normoxia. Analysis of the mode of action of TNF-α revealed the accumulation of hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($^{\bullet}OH$). Antioxidants purported as prototypical scavengers of H_2O_2 and $^{\bullet}OH$, attenuated TNF-α-induced HIF-1α activation, and blockading NADPH-oxidase by scavenging $O_2^{-\bullet}$ reduced the activity of HIF-1α. Inhibition of the mitochondrion complex I abrogated TNF-α-dependent activation of HIF-1α. Interrupting the respiratory chain reversed the excitatory effect of TNF-α on HIF-1α. These results indicate a non-hypoxic pathway mediating cytokine-dependent regulation of HIF-1α in a ROS-sensitive mechanism.

Key words: Antioxidant; Hypoxia-inducible factor-1α; Mitochondrion; Oxygen sensing; Reactive oxygen species; Tumor necrosis factor-α

1. Introduction

The hypoxia-inducible factor-1α (HIF-1α), identified as a DNA-binding activity expressed under hypoxic conditions, has its stability and activity increased exponentially on lowering pO_2 over physiologically relevant ranges [1]. The ubiquitous activation of HIF-1α is thus consistent with the significant role that this transcription factor plays in coordinating adaptive responses to hypoxia.

HIF-1 is a heterodimeric transcription factor consisting of α and β subunits. The aryl hydrocarbon receptor nuclear translocator (HIF-1β) can dimerize with the aryl hydrocarbon receptor as well as with HIF-1α [1]. Whereas HIF-1β is constitutively expressed under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-proteasome system [2]. However, under hypoxic conditions, HIF-1α is stabilized and accumulated, allowing the heterodimer to translocate and bind specific promoter moieties of genes encoding erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic transporters [3–5] and inflammatory mediators [6–8]. Therefore, HIF-1α may play an important role not only in regulating the transcription of pO_2-controlled genes and energy homeostasis, but also influence an immune response. However, the mechanism of pro-inflammatory cytokine-dependent regulation of the translocation/activation of HIF-1α is currently unknown.

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine with a plethora of multiple actions on cellular metabolism. TNF-α has been implicated in the pathogenesis of respiratory distress syndrome and pulmonary edema [9], in regulating apoptosis signaling and pathways [10,11] and in governing gene expression [11,12]. In the lung, TNF-α is produced primarily by activated pulmonary macrophages, however, evidence suggested that it is produced in fibroblasts and epithelial cells [13–16]. Furthermore, the alveolar epithelium has been implicated in regulating the immunological functions of the lung by controlling pro-inflammatory gene expression [17,18]. In the present investigation, we studied the role that TNF-α plays in regulating HIF-1α translocation/activation and subsequently explored the underlying molecular pathway implicated.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise indicated, chemicals of the highest analytical grade were purchased from Sigma-Aldrich Co. Experimental procedures were approved under the Animals Act, 1986 (UK).

2.2. Primary cell cultures of alveolar epithelia

Fetal alveolar type II epithelial cells were isolated from the lungs of fetuses of pregnant rats, essentially as reported elsewhere [19,20]. The adenylate energy charge, an index of cell viability and competence, remained ≥0.7 and transepithelial monolayer resistance (R_t) was monitored constantly at ≥250–350 Ω cm^2 [19].

2.3. Administration of recombinant murine (rm) TNF-α and analysis of HIF-1α translocation/activation

Epithelial cells were exposed to rmTNF-α (10 ng/ml) for indicated time periods. Nuclear extracts were prepared and Western/DNA-binding activity were subsequently performed, essentially as described elsewhere [19,20]. Nuclear proteins (20–25 μg) were resolved over SDS–PAGE (7.5%) gels at room temperature and mouse monoclonal IgG_1 anti-HIF-1α (Santa Cruz Biotechnology, UK) antibody was employed for primary detection, followed by secondary detection in the presence of streptavidin–horseradish peroxidase conjugate and then visualized by chemiluminescence [19,20]. In addition, nuclear extracts were analyzed for HIF-1α DNA-binding activity by electrophoretic mobility shift assay (EMSA), as detailed previously [19,20].

2.4. Assessment of intracellular reactive oxygen species (ROS) accumulation with rmTNF-α

For the determination of H_2O_2, cell supernatants were collected and centrifuged at 2000×*g* for 5 min at 4°C, and then treated with 1 M NaOH. The change in absorbancy at 600 nm was monitored against phenol red solution (PRS) containing (in mM): 140 NaCl,

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10 K_3PO_4, 5.5 glucose, 0.28 phenol red, and 20 U/ml horseradish peroxidase [15,16,21,22]. Standard curves using PRS and H_2O_2 were prepared (0–100 μM), and results extrapolated from the linear regression were converted to nmol/mg protein. For the quantification of $O_2^{-\bullet}$, supernatant was discarded after 24 h of culture, and cells were then reincubated with rmTNF-α (37°C) in the presence of 80 μM ferricytochrome *c* (fc) suspended in Hank's buffered salt solution. The amount of $O_2^{-\bullet}$ released was determined by measuring the absorbancy at 550 nm against blanks containing fc and superoxide dismutase (300 U/ml) [15,16,21,22]. For the determination of $^{\bullet}OH$, cells were cultured at 10^5/well in flat-bottomed microtiter plates, then washed with sterile saline, and culturing was continued in Krebs–Ringer solution containing 50 μM dihydrorhodamine and rmTNF-α. Fluorescence was measured at excitation/emission wavelengths of 485/535 nm [15,16].

2.5. Pre-treatment with selective antioxidants prior to exposure to exogenous rmTNF-α

In order to investigate the possibility of ROS as signaling messengers in rmTNF-α-mediated regulation of HIF-1α, selective antioxidants were employed. This choice was based on the antioxidant potency and specificity: dimethyl sulfoxide (DMSO) is a scavenger of $^{\bullet}OH$ [23,24]; 1,3-dimethyl-2-thiourea (DMTU) scavenges H_2O_2 and $^{\bullet}OH$ [23–25]; and 4′-hydroxy-3′-methoxy-acetophenone (HMAP) is a NADPH-oxidase inhibitor which neutralizes endogenous $O_2^{-\bullet}$ [26]. Cells were pre-treated for 2 h with DMSO, DMTU or HMAP, then exposed to rmTNF-α (10 ng/ml) for 30 min. Nuclear extracts were prepared and DNA-binding activity of HIF-1α was subsequently performed. Separately, to determine the level of participation of mitochondrial ROS, cells were pre-treated with diphenylene iodonium (DPI), an inhibitor of the mitochondrial complex I [27], or KCN, an inhibitor of the respiratory chain, followed by exposure to rmTNF-α and assessment of the DNA-binding activity. In independent experiments, to give a direct evidence of a ROS-generating system through activation by rmTNF-α, cells were pre-treated with either HMAP or DPI as above, then exposed to rmTNF-α (10 ng/ml) for 30 min, followed by assessment of intracellular ROS accumulation.

2.6. Statistical analysis and data handling

Data are the means and the error bars the S.E.M. Statistical analysis of the difference in mean separation was performed by one-way analysis of variance, followed by post-hoc Tukey's test, and the a priori level of significance at 95% confidence level was considered at $P \leq 0.05$.

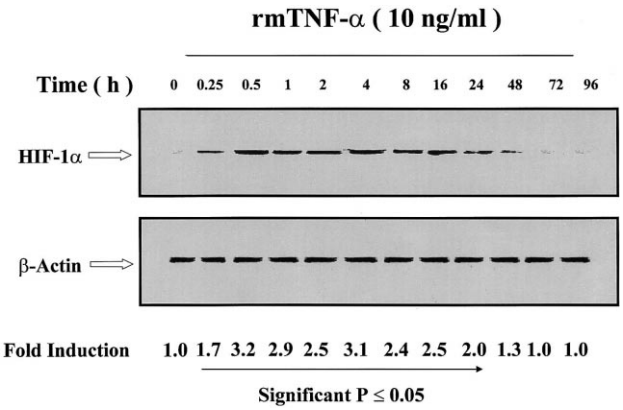

Fig. 1. TNF-α-mediated regulation of HIF-1α stabilization/translocation. TNF-α (10 ng/ml) induced, in a time-dependent manner, stabilization and nuclear accumulation of HIF-1α under normoxia. HIF-1α protein was detected at 15 min, maximized at 0.5–4 h, and still persistently abundant until 24 h, thereafter declining. β-Actin was used as an internal reference for semi-quantitative loading per lane. $n = 3$, which represents the number of independent experiments.

3. Results

3.1. The role of rmTNF-α in regulating the stability and nuclear translocation of HIF-1α

Fig. 1 displays the effect of rmTNF-α on the nuclear localization of the HIF-1α subunit under normoxic conditions.

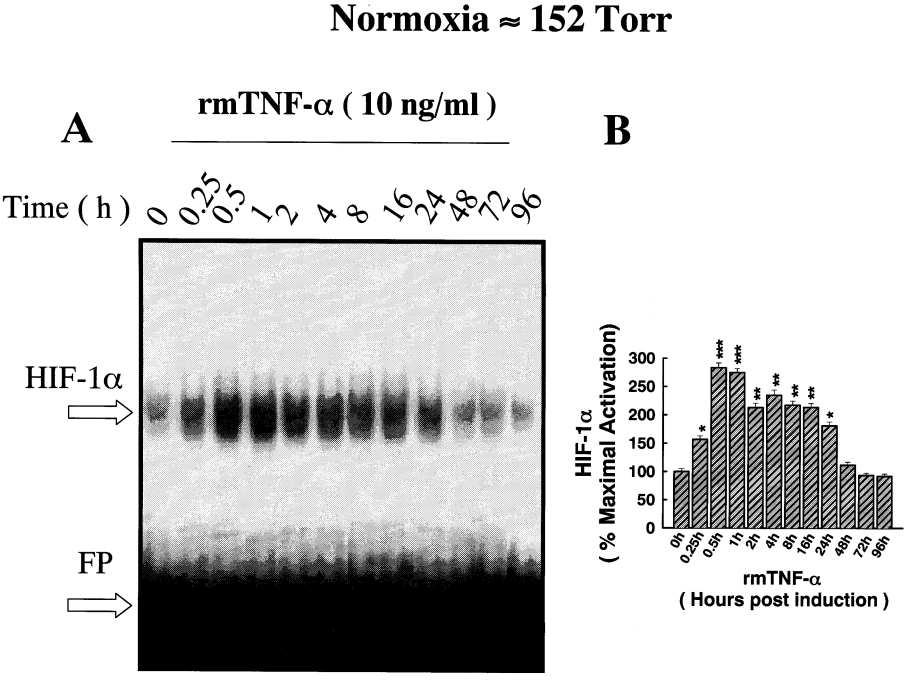

Fig. 2. The effect of TNF-α on HIF-1α activation. A: TNF-α increased the DNA-binding activity of HIF-1α in a time-dependent manner. HIF-1α activity was detectable at 15 min, maximized at 0.5–1 h, and continued to be active until 24 h, thereafter declining. The faster migrating probe (FP) indicates the free, unbound probe, and the upper-shifted band is that of the HIF-1α/DNA complex. B: Histogram analysis of the gel-shifted band as determined by phosphorimaging. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as compared with control in the absence of rmTNF-α. $n = 3$, which represents the number of independent experiments.

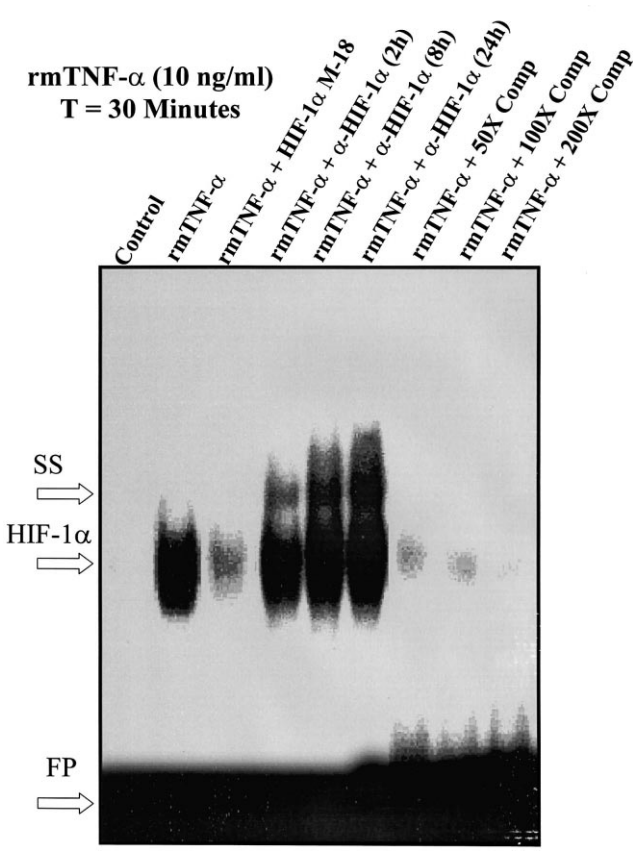

Fig. 3. Specificity of HIF-1α gel-shifted band in response to rmTNF-α. No detectable activity was recorded for HIF-1α in control nuclear samples containing no rmTNF-α. Addition of rmTNF-α (10 ng/ml) induced the activity of HIF-1α. Incubation with the mutant oligonucleotide (HIF-1α, M-18) abolished the shifted band. Pre-incubation with α-HIF-1α antibody caused the appearance of a further retarded band, with time effect evident ranging from 2–24 h. The cold competitor (COMP) abolished the specific band of HIF-1α obtained due to rmTNF-α. The faster migrating probe (FP) indicates the free, unbound probe, the upper-shifted band is that of the HIF-1α/DNA complex, and SS indicates the super shift. $n=3$, which represents the number of independent experiments.

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HIF-1α accumulation increased significantly as early as 15 min post-addition of rmTNF-α and continued to increase exponentially (maximizing at 0.5–4 h) until 24 h, thereafter declining (Fig. 1). The housekeeping protein, β-actin, was used as an internal reference for semi-quantitative loading per lane (Fig. 1).

3.2. The effect of rmTNF-α on the nuclear activation of HIF-1α

In association with its ability to up-regulate HIF-1α nuclear translocation, rmTNF-α induced the DNA-binding activity of this transcription factor (Fig. 2A). EMSA analysis detected HIF-1α activity as early as 15 min after addition of rmTNF-α, became maximally active at around 0.5–1 h, persistently active until 24 h, thereafter declining (Fig. 2A). Histogram analysis of the gel-shifted bands as performed by phosphorimaging is displayed in Fig. 2B. In order to verify

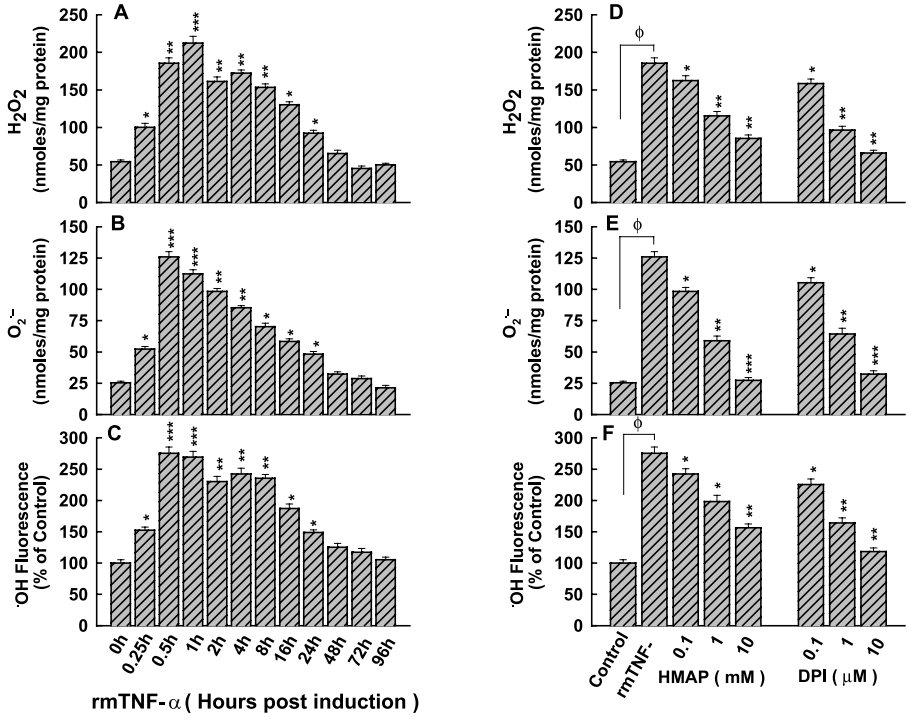

Fig. 4. Analysis of ROS accumulation in response to rmTNF-α and the role of selective antioxidants. A: H_2O_2 began to increase at 15 min and continued to be significantly higher than control until 24 h, thereafter declining. B: $O_2^{-\bullet}$ appeared at 15 min and continued to be higher than control until 24 h, thereafter declining. C: The concentration of $^{\bullet}OH$ (fluorescence) began to increase at 15 min and continued to be significantly higher than control until 24 h, thereafter declining. D: HMAP and DPI pre-treatments reduced accumulation of H_2O_2 in response to rmTNF-α. E: HMAP and DPI reduced accumulation of $O_2^{-\bullet}$ in response to rmTNF-α in a dose-dependent manner. F: HMAP and DPI mildly reduced accumulation of $^{\bullet}OH$ in response to rmTNF-α. $^{\phi}P<0.05$, as compared with control; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, as compared with control in the absence or presence of rmTNF-α. $n=4$, which represents the number of independent experiments run in duplicate.

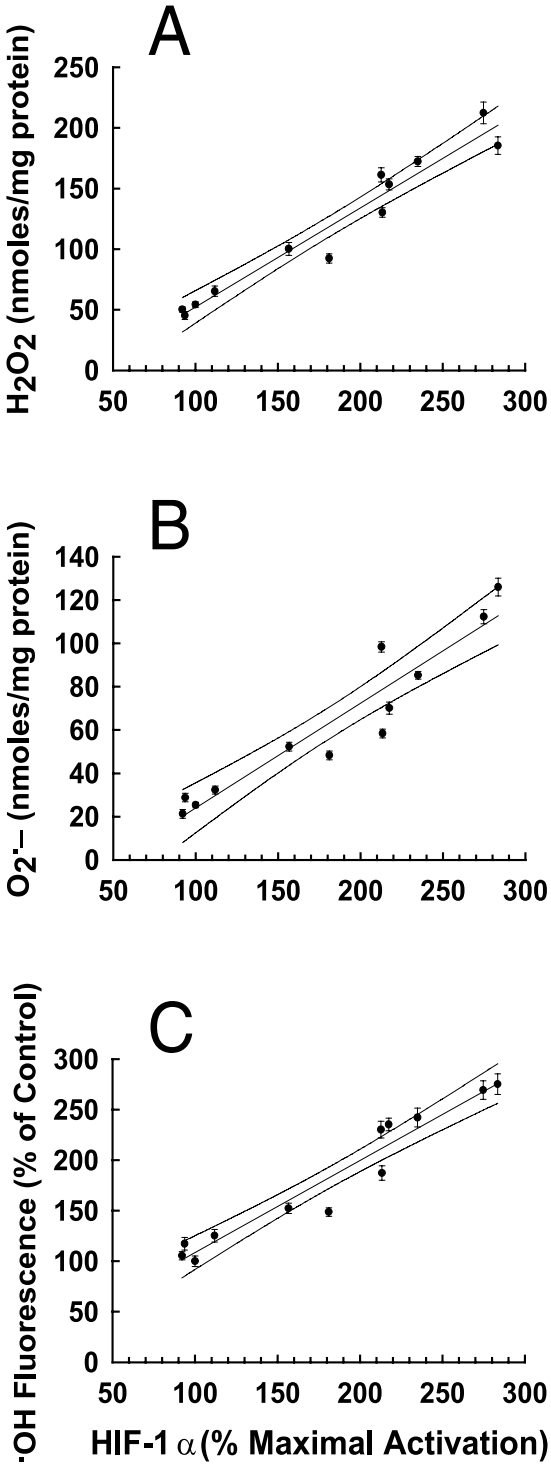

Fig. 5. Subset correlation analysis of ROS on the activation of HIF-1α. A: Correlation with H_2O_2. B: Correlation with $O_2^{-\bullet}$. C: Correlation with $^{\bullet}OH$. The regression wizard lines represent the degree of confidence at 95% limit. $n = 3$, which represents the number of independent experiments.

the specificity of the shifted bands corresponding to HIF-1α, supershift analysis was performed. As shown in Fig. 3, rmTNF-α had no effect on the binding activity of HIF-1α (control); in contrast, rmTNF-α induced the activity of HIF-1α. Pre-incubating nuclear extracts with a specific monoclonal antibody raised against the α subunit induced a supershift retarded band relative to that induced by rmTNF-α alone (Fig. 3). Nuclei incubated with α-HIF-1α for 2 h showed a faint supershifted band, whereas pre-incubation for 8 or 24 h exhibited a clearer supershifted band (Fig. 3). To further delineate the specificity of HIF-1α bands, extracts were incubated in the presence of the mutant oligonucleotide versus the wild-type, where M-18 is shown to suppress the effect of rmTNF-α. Addition of cold competitor abrogated rmTNF-α-induced HIF-1α DNA-binding activity (Fig. 3).

3.3. The effect of rmTNF-α on intracellular accumulation of ROS

The underlying mechanism of the mode of action of rmTNF-α was investigated. TNF-α induced in a time-dependent manner the accumulation of H_2O_2 (Fig. 4A), $O_2^{-\bullet}$ (Fig. 4B) and $^{\bullet}OH$ (Fig. 4C). In order to assess the likely source of ROS in response to rmTNF-α, cells were pre-treated with HMAP or DPI prior to exposure to rmTNF-α for 30 min. As shown in Fig. 4, HMAP and DPI reduced H_2O_2 accumulation (Fig. 4D), but to a lesser extent than $O_2^{-\bullet}$ (Fig. 4E). The effect of HMAP on $^{\bullet}OH$ was milder than that of DPI (Fig. 4F). These results indicated that both the membrane-bound NADPH oxidase and the mitochondrial complex are differentially involved as potential resources for ROS in response to rmTNF-α. Correlation studies were performed to determine the degree of statistical confidence between intracellular accumulation of ROS in response to rmTNF-α and the activity of HIF-1α. Fig. 5 shows that there is a positive correlation between H_2O_2 (Fig. 5A), $O_2^{-\cdot}$ (Fig. 5B) and ($^{\bullet}OH$) accumulation and HIF-1α activation (Fig. 5C).

3.4. The effect of selective antioxidants on rmTNF-α-induced HIF-1α activation

To confirm statistical correlation observed, we investigated the role of selective antioxidants and determined the level of involvement of the mitochondrial complex. The effect of DMSO on rmTNF-α-dependent activation of HIF-1α is shown in Fig. 6A ($IC_{50} = 1.12 \pm 0.08\%$), that for DMTU in Fig. 6B ($IC_{50} = 0.67 \pm 0.05$ mM), that for HMAP in Fig. 6C

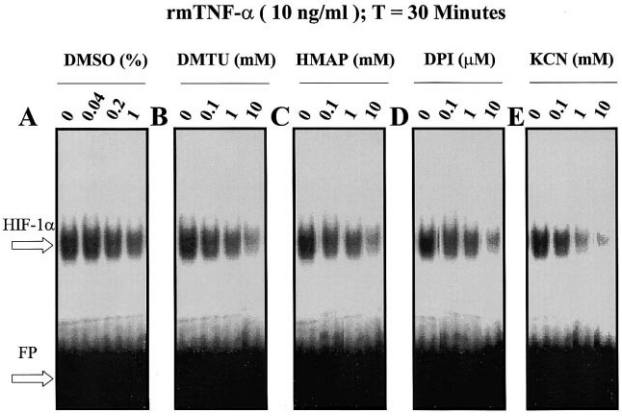

Fig. 6. Analysis of the effect of antioxidants and mitochondrial respiration inhibitors on rmTNF-α-mediated HIF-1α activation. A: The inhibitory effect of DMSO (effective a doses ≥0.2%). B: The inhibitory effect of DMTU (effective a doses ≥0.1 mM). C: The inhibitory effect of HMAP (effective a doses ≥0.1 mM). D: The inhibitory effect of DPI (effective a doses ≥0.1 μM). E: The inhibitory effect of KCN (effective a doses ≥0.1 mM). $n = 3$, which represents the number of independent experiments. FP: Faster migrating probe.

($IC_{50} = 0.62 \pm 0.07$ mM), that for DPI in Fig. 6D ($IC_{50} = 0.64 \pm 0.10$ μM) and that for KCN in Fig. 6E ($IC_{50} = 0.52 \pm 0.10$ mM).

4. Discussion

The present investigation revealed a novel role for ROS signalling in mediating the effect of TNF-α on HIF-1α stabilization, translocation and activation. Despite that HIF-1α was recognized as a transcriptional activity prevailing under hypoxic conditions that transiently gets degraded with ascending ΔpO_2 [1,31], ROS mediating the regulation of HIF-1α stabilization, translocation and activation have recently emerged as potential regulatory mechanisms [27–31]. Consistent with this notion, we reported for the first time a non-hypoxic pathway mediating the effect of TNF-α regulating the translocation/activation of HIF-1α in an ROS-dependent mechanism. Our results have clearly negated the hypothesis that hypoxia is the only major player in HIF-1α regulation and that this pathway mediated by TNF-α, for the moment specific to alveolar epithelial cells, should confer a major role in controlling the mechanism mediating HIF-1α regulation under normoxia.

The concept has been put forward that ROS events are master regulators of HIF-1α, however, the signalling pathway implicated awaits clarification. On the mechanism of the non-hypoxic regulation of HIF-1α, Chandel et al. [27,28] reported a role for mitochondrial ROS generated at complex III and that depletion of mitochondrial genome reversed ROS-mediated induction of HIF-1α. Furthermore, it has been reported that HIF-1α-dependent transcriptional activity mediating VEGF induction has defined an hypoxia-independent mechanism regulating vascular remodeling [29]. Further afield, recent evidence suggested that reactive nitrogen species regulate the stability/activation of HIF-1α. For instance, it was shown that expression of nitric oxide (NO) synthase induced HIF-1α accumulation, which underscored the role of NO as an intracellular activator of this transcription factor [30,31].

The molecular pathways governed by cytokines mediating HIF-1α regulation are not understood. Recently, Hellwig-Bürgel et al. [7] reported a role for interleukin (IL)-1β and TNF-α as mediators in stimulating the DNA-binding activity of HIF-1α, concluding that it is likely to be involved in modulating gene expression during inflammation. The augmented activity of HIF-1α due to IL-1β was attributed to increased HIF-1α protein abundance, whereas the effect of TNF-α was proposed to be due to concomitant activation of certain proteins forming part of the activated complex [7,32]. Moreover, cAMP responsive element-binding protein has been implicated in augmenting HIF-1α activity within its site in the EPO gene enhancer [33]. Despite the observation that there was failure at demonstrating any effect of inflammatory cytokines on cAMP levels and PKA activity, it was noted that the addition of cAMP analogues prevented the suppression of EPO mediated by cytokines [34].

Amongst the cytokines most known for their inflammatory actions, perhaps TNF-α is the one exhaustively investigated. TNF-α is a potent inflammatory cytokine, which exerts its pleiotropic activities through ligand-induced cross-linking of specific receptors, virtually present in almost all cell types [35]. TNF-α released in the inflammatory milieu transiently activates neutrophils and macrophages, thereby causing them to release $O_2^{-\bullet}$ as a consequence of the activation of NADPH oxidase [36]. This oxidative burst features a rapid release of ROS and is a crucial part of the defense mechanism against invading microbial pathogens and tumor cell metastasis. Although TNF-α is primarily produced by macrophages, growing evidence suggests that other cell types release TNF-α and other inflammatory mediators, thereby amplifying and boosting the inflammatory reaction [15,16,18,37]. In this respect, it has been reported that TNF-α up-regulates intracellular accumulation of ROS [14]. More recently, Corda et al. [38] specifically defined a role of the mitochondrion in TNF-α-mediated ROS generation. Our results, indicating a potential role for TNF-α in accumulating intracellular ROS, jibe with the notion that this cytokine mediates its effect through an ROS-dependent mechanism. On the likely source of ROS, we report the convergence of two possible pathways: (i) the membrane-bound NADPH oxidase and (ii) the respiratory chain of the mitochondrial complexes. Both resources are likely to be implicated in mediating the effect of TNF-α on HIF-1α induction. The latter mechanism is rather directly involved since blockading the mitochondrial complex I and respiratory chain abrogated TNF-α-dependent HIF-1α activation. Furthermore, we provided evidence that different ROS species are implicated in mediating the effect of TNF-α on HIF-1α translocation/activation. The observation that a non-hypoxic pathway mediated by ROS generated within the mitochondrion complex regulates cytokine-dependent HIF-1α induction is rather unequivocal since DPI, an inhibitor of complex I which blockades the conversion of ubiquinone → ubiquinol [27,28,31], almost totally abrogated TNF-α-induced HIF-1α activation, suggesting a vital role for mitochondrial ROS.

The present investigation has thrown a shadow of light on the mechanism of action of TNF-α mediating its inductive effect on HIF-1α. The results could be highlighted as follows: (i) TNF-α allowed the stabilization and nuclear translocation of HIF-1α under normoxic conditions; (ii) this effect was associated with inducing HIF-1α DNA-binding activity; (iii) TNF-α augmented intracellular accumulation of ROS; (iv) selective antioxidants attenuated TNF-α-mediated activation of HIF-1α; and (v) blockading the mitochondrion complex I and its respiratory chain abrogated TNF-α-dependent HIF-1α activation. It is concluded that cytokine-mediated regulation of HIF-1α stabilization, translocation and activation requires a non-hypoxic, ROS-sensitive mechanism.

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