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Recombinant human interleukin (IL)-1beta-mediated regulation of hypoxia-inducible factor-1alpha (HIF-1alpha) stabilization, nuclear translocation and activation requires an antioxidant/reactive oxygen species (ROS)-sensitive mechanism

Abstract

Cytokine-mediated regulation of hypoxia-inducible factor-1alpha (HIF-1alpha) non-hypoxic stabilization, translocation and activation is not well characterized. Furthermore, evidence that reactive oxygen species (ROS) signaling mediates interleukin (IL)-1beta-dependent regulation of HIF-1alpha has yet to be ascertained in alveolar epithelial cells. Recombinant human IL-1beta induced, in a time-dependent manner, the nuclear translocation of HIF-1alpha, an effect associated with up-regulating the activity of this transcription factor under normoxic conditions. In addition, analysis of the mode of action of IL-1beta revealed a novel induction of intracellular ROS, including hydrogen peroxide (H2O2), the superoxide anion (O2-*) and the hydroxyl radical (*OH). The antioxidants, dimethyl sulfoxide (DMSO) and 1,3-dimethyl-2-thiourea (DMTU), purported to be prototypical scavengers of H2O2 and *OH, attenuated, in a dose-dependent manner, IL-1beta-induced HIF-1alpha nuclear translocation and activation. The NADPH-oxidase inhibitor, 4'-hydroxy-3'-methoxy-acetophenone (HMAP), which may affect mitochondrial ROS production, attenuated IL-1beta-mediated nuclear translocation and activation of HIF-1alpha. Inhibition of the mitochondrion complex I nicotinamide adenine dinucleotide phosphate-dependent oxidase by diphenylene iodonium (DPI), which blocks the conversion of ubiquinone = › ubiquinol, abrogated IL-1beta-dependent nuclear translocation and activation of HIF-1alpha. Similarly, interrupting the respiratory chain with potassium cyanide reversed the excitatory effect of IL-1beta on HIF-1alpha nuclear translocation and activation. These results indicate that a non-hypoxic pathway mediates cytokine-dependent regulation of HIF-1alpha translocation and activation in a ROS-sensitive mechanism.

Full text

ABBREVIATIONS

AEBSF: 4-(2-aminoethyl)-benzene sulfonyl fluoride-HCl

ARNT: aryl hydrocarbon receptor nuclear translocator

BSA: bovine serum albumin

CREB: cAMP-responsive element binding protein

DPI: diphenylene iodonium

DHR: dihydrorhodamine

DMSO: dimethyl sulfoxide

DMTU: 1,3-dimethyl-2-thiourea

DTT: dithiothreitol

EMSA: electrophoretic mobility shift assay

EPO: erythropoietin

Fc: ferricytochrome c

GLUT: glucose transporter

HBSS: hankappas' balanced salt solution

HRP: horseradish peroxidase

H_2O_2: hydrogen peroxide

HMAP: 4'-hydroxy-3'-methoxy-acetophenone

$^{*}OH$: hydroxyl radical

HIF-1alpha: hypoxia-inducible factor-1alpha

IL: interleukappain

NO: nitric oxide

PAS: per-ARNT-Sim

PRS: phenol red solution

PBS: phosphate-buffered saline

PKAPPAA: protein kappainase A

RNS: reactive nitrogen species

ROS: reactive oxygen species

rhIL-1beta: recombinant human interleukappain-1beta

O_2^{-*}: superoxide anion

SOD: superoxide dismutase

R_t: transepithelial resistance

VEGF: vascular endothelial growth factor.

INTRODUCTION

The stability and activity of hypoxia-inducible factor-1alpha (HIF-1alpha), first identified as a DNA-binding activity expressed under hypoxic conditions [1], increase exponentially when pO_2 is lowered [2-4]. The ubiquitous activation of HIF-1alpha is thus consistent with the significant role that this transcription factor plays in coordinating adaptive responses to hypoxia [2-4]. HIF-1 is a heterodimeric complex consisting of alpha and beta subunits. The aryl hydrocarbon receptor nuclear translocator (ARNT; HIF-1beta) can dimerize with the aryl hydrocarbon receptor as well as with HIF-1alpha, because both contain helix-loop-helix and PAS ("Per-ARNT-Sim") domains [1-4]. Whereas HIF-1beta is constitutively expressed under normoxic conditions, HIF-1alpha is rapidly degraded by the ubiquitin-proteasome system [5-8]. However, under hypoxic conditions, HIF-1alpha protein stabilizes and accumulates, thus allowing the heterodimer to translocate to the nucleus and to bind specific promoter moieties of selective genes encoding erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes and glucose transporters (GLUT) [9, 10], as well as cytokappaines and other inflammatory mediators [11-14].

Adaptive responses to hypoxia, therefore, involve the regulation of gene expression by HIF-1alpha, at least in part, whose expression, stability and

transcriptional activity are reported to increase exponentially on lowering pO_2 [2-4, 7-9]. During hypoxia, multiple systemic responses are induced, including angiogenesis, erythropoiesis and glycolysis. HIF-1alpha is a crucial mediator for increasing the efficiency of O_2 delivery through EPO and VEGF [2-4]. A well-controlled process of adaptation parallels this mechanism with decreased O_2 availability through expression and activation of glucose transporters and glycolytic enzymes [2-4, 8]. EPO, for example, is responsible for increasing the blood O_2-carrying capacity by stimulating erythropoiesis, VEGF is a transcriptional regulator of vascularization and glycolytic transporters and enzymes increase the efficiency of anaerobic generation of ATP, the vital biological currency. It is expected that any reduction of tissue oxygenation *in vivo* and *in vitro* would, therefore, provide a mechanistic stimulus for a graded and adaptive response mediated by HIF-1alpha [2-4, 7, 8].

HIF-1alpha, therefore, may play an important role not only in regulating the transcription of pO_2-controlled genes and energy homeostasis, but also in influencing immune responses. However, the mechanism of cytokappaine-dependent regulation of the translocation and activation of HIF-1alpha is currently unkappanown. Cytokappaines act as major participants in mediating molecular responses in physiology and pathophysiology [14-28]. There is accumulating evidence, moreover, suggesting that the conventially kappanown "pro-inflammatory" cytokappaines, including interleukappain (IL)-1beta, act as O_2-sensitive mediators, indicating that the alveolar epithelium possesses the potential to integrate O_2-linkappaed pathways mediated by cytokappaines *via* a ROS-dependent mechanism [18-25]. Direct contact of the epithelium with dynamic fluctuations in pO_2 at the alveolar space-blood barrier junction during pulmonary-based ventilation is likappaely therefore, to up-regulate the transcription, biosynthesis and mobilization of cytokappaines. This notion is strengthened by the evidence implicating the epithelium in a front-line defense strategy activated in preparation for birth into an O_2-rich environment [6-8, 18-28]. Furthermore, the generation of ROS might induce pulmonary damage; however, moderate oxidative stress can induce a signaling mechanism mediated, at least in part, by O_2-responsive cytokappaines [18-30]. ROS, for instance, can induce pro-inflammatory cytokappaine biosynthesis and this response can be abrogated by selective antioxidants, suggesting an integral role of endogenous ROS [18, 19, 31, 32]. As such, cytokappaines could form a pivotal linkappa in ROS-dependent pathways leading to the activation of redox-sensitive transcription factors such as HIF-1alpha, whose up-regulation

determines the specificity of cellular responses to oxidative stress [28, 29, 33, 34].

IL-1beta is a pleiotropic cytokappaine with multiple actions on cellular metabolism [35-41]. This cytokappaine has been implicated in the pathogenesis of human diseases [9, 15, 19, 21, 25, 30, 35, 38, 40], in regulating apoptosis signaling and pathways [13, 16, 34, 36, 39] and in governing gene expression and activation [10, 11, 14, 17, 18, 28, 33, 37]. In the lung, IL-1beta is primarily produced by pulmonary macrophages; however, recent evidence suggested that this cytokappaine is also synthesized and transcribed in fibroblasts and epithelial cells [6, 7, 13-18]. Since cytokappaine-mediated regulation of HIF-1alpha during normoxia is not well understood, the present investigation attempted to study the role that IL-1beta plays in regulating HIF-1alpha non-hypoxic translocation and activation in alveolar epithelial cells. In addition, the underlying molecular pathway potentially involving signaling cofactors mediating the effect of IL-1beta was subsequently identified and explored.

MATERIALS AND METHOD

Materials

Unless indicated otherwise, chemicals of the highest analytical grade were purchased from Sigma-Aldrich Co. (Dorset, England, UKAPPA). Recombinant human IL-1beta (rhIL-1beta) was purchased from the National Institute for Biological Standards and Control (NIBSC, England, UKAPPA), where its activity was authenticated (1 mug ± 200,000 IU) as supplied by the manufacturer. All experimental procedures involving the use of live animals were reviewed and approved under the Animals (Scientific Procedures) Act, 1986 (UKAPPA).

Primary cell cultures of alveolar epithelia

Fetal alveolar type II epithelial cells were isolated from the lungs of Sprague-Dawley rat fetuses, as reported elsewhere [6, 7, 18-20]. Briefly, fetal rats were removed by caesarian section at day 19 of gestation (term = 22 days). The lungs were excised, teased free from heart and upper airway tissue, finely minced and then washed free of erythrocytes using sterile, chilled Mg^{2+} - and Ca^{2+} - free Hankappas' balanced salt solution (HBSS; 0.5 ml/fetus). The cleaned lung tissue was re-suspended in 1 ml/fetus HBSS containing trypsin (0.1 mg/ml),

collagenase (0.06 mg/ml) and DNase I (0.012% w/v) and was agitated at 37° C for 20 min. The solution then was centrifuged at 100 *g* for 2 min to remove undispersed tissue, the supernatant was decanted into a sterile tube and an equal volume of Dulbecco's modified Eagle medium (DMEM), with 10% (v/v) fetal calf serum (FCS) was added to the supernatant. After passing the supernatant through a 120 mum pore sterile mesh, the filtrate was centrifuged at 420 *g* for 5 min, the pellet re-suspended in 20 ml DMEM/FCS and the cells were placed into a T-150 culture flaskappa for 1 hour at 37° C enabling fibroblasts and non-epithelial cells to adhere. Unattached cells were washed three times by centrifugation at 420 *g* for 5 min each and seeded onto 24 mm diameter Transwell-clear permeable supports (Costar; 0.4 mum pore size) at a density of 5×10^6 cells per filter and then were allowed to adhere overnight at normoxia (21% O_2/5% CO_2), at 37° C. DMEM/FCS was exchanged for 4 ml of pre-equilibrated serum-free PC-1 medium (Biowhittakappaer, MD, USA) after 24 hours and cells were maintained under these conditions until the experiment. The adenylate energy charge, an index of cell viability and competence, remained at >= 0.7 and transepithelial monolayer resistance (R_t) was monitored constantly at >= 250-350 ômega cm^2 [6, 7].

*Administration of exogenous rhIL-1*beta *and analysis of HIF-1*alpha *translocation/activation*

Epithelial cells, grown during normoxia, were exposed to exogenous rhIL-1beta (10 ng/ml) for the indicated time periods (0-96 hours). Nuclear extracts were prepared and western/DNA-binding activity assays were performed subsequently, as described previously [6, 7]. Briefly, filters were washed twice in 5 ml ice-cold, pre-equilibrated phosphate-buffered saline (PBS) and cells ($1-2 \times 10^7$) were collected and centrifuged at 420 *g* for 5 min at 4° C. Nuclei were released by re-suspending the pellet in 250 mul buffer A containing (in mM): 10 Tris-HCl (pH 7.8), 10 KAPPACl, 2.5 NaH_2PO_4, 1.5 $MgCl_2$, 1 Na_3VO_4, 0.5 dithiothreitol (DTT), 0.4 [4-(2-aminoethyl)-benzene sulfonyl fluoride-HCl] (AEBSF), and 2 mug/ml each of leupeptin, pepstatin A and aprotinin. The suspension was left on ice for 10 min followed by a 45-second homogenization at a moderate speed. Nuclei were collected by centrifuging the slurry at 4,500 *g* for 5 min at 4° C and re-suspending in 100 mul buffer B (buffer A adjusted to (in mM): 20 Tris-HCl (pH 7.8), 420 KAPPACl, 20% (v/v) glycerol). The supernatant formed is termed the cytosolic extract. The nuclei were then lysed at 4° C for 30 min with gentle agitation, the debris cleared by centrifugation at 10,000 *g* for an additional 30

min at 4° C and the supernatant frozen in liquid nitrogen and stored at - 70° C until use. In all cases, protein content was determined by the Bradford method using bovine serum albumin (BSA) as a standard. Nuclear proteins (20-25 mug) were resolved over SDS-PAGE (7.5%) gels at room temperature, blotted onto nitrocellulose membrane, and non-specific binding sites were subsequently blockappaed. Mouse monoclonal IgG_1 anti-HIF-1alpha antibody (Santa Cruz Biotechnology, CA, USA) was employed for primary detection. Anti-rabbit Ig-biotinylated antibody (Amersham Life Science, USA) was employed for secondary detection followed by the addition of streptavidin-HRP conjugate and visualized on film by chemiluminescence. beta-actin standard was used as an internal reference for semi-quantitative loading in parallel lanes for each variable. Western blots were scanned by NIH MagiScanII and subsequently quantitated by UN-Scan-IT automated digitizing system (version 5.1; 32-bit), and the ratio of the density of the band to that of beta-actin was subsequently performed [6, 7].

Nuclear extracts were analyzed for HIF-1alpha DNA binding activity by electrophoretic mobility shift assay (EMSA) [6, 7, 20, 23]. Custom deoxy-oligonucleotide probe sequences were purchased from Sigma-Genosys, UKAPPA: HIF-1alpha (consensus sequence), W-18: 5'-GCCCTACGTGCTGTCTCA-3' (binding sequence underlined) (3-bp missense control, M-18: 5'-GCCCTAAAAGCTGTCTCA-3'). Gel-purified double-stranded DNA was end-labeled with [gamma^{32}P]-ATP (NEN Life Sciences Products, UKAPPA). Identical amounts of radioactive probe (1-2 x 10^4 counts/min) were added to binding reactions containing 1-5 mug nuclear extracts to a final volume of 40 mul in DNA binding buffer (20 mM HEPES (pH 7.9); 1 mM $MgCl_2$; 4% Ficoll). Reaction mixtures were incubated for 30 min at 25° C before separating on non-denaturing 4% polyacrylamide gels at room temperature and subjected to electrophoresis with 1:10 5X Tris-Borate-EDTA buffer. A non-specific competitive polydeoxyinosinic-deoxycytidylic acid [poly(dI-dC)] (Amersham Pharmacia Biotech, USA) was added to reaction mixtures after addition of the labeled probe. Where indicated, non-labeled oligonucleotide competitor was added in 50-, 100- and 200-fold molar excess immediately prior to addition of a radiolabeled probe of the same sequence. For super-shift experiments, a specific antibody for HIF-1alpha (monoclonal IgG anti-HIF-1alpha; Novus Biologicals, UKAPPA) was used at 2 mug/reaction. The antibodies were incubated with nuclear extracts prior to the addition of probe (W-18) for 2, 8 or 24 hours at 4° C, and then processed as above. Gels were transferred to ion-exchange chromatography paper, vacuum

dried and then electronically visualized on a Packappaard Instant phosphorimager. Specific quantitation of the corresponding DNA gel shift bands was performed with phosphorimaging [6, 7].

*Assessment of intracellular reactive oxygen species (ROS) accumulation with rhIL-1*beta

Analysis of hydrogen peroxide (H_2O_2). Phenolsulfon phthalein (phenol red) containing horseradish peroxidase (HRP; 20 U/ml) was added to monolayers. Cell supernatants were collected after the addition of IL-1beta, centrifuged at 2,000 *g* for 5 min at 4° C, and then treated with 1 M NaOH to terminate the reaction. The change in absorbency at 600 nm was monitored against phenol red solution (PRS) containing (in mM): 140 NaCl, 10 $KAPPA_3PO_4$, 5.5 glucose, 0.28 phenol red, and 20 U/ml HRP [18, 19, 42]. Standard curves using PRS and H_2O_2 were prepared (0-100 muM), and results extrapolated from the linear regression were converted to nmoles/mg protein.

Analysis of superoxide anion (O_2^{-*}). For the quantification of O_2^{-*} release, the supernatant was discarded after 24 hours of culture under atmospheric oxygen tension, and cells were reincubated with rhIL-1beta (37° C) in the presence of 80 muM ferricytochrome c (fc) suspended in HBSS. The amount of O_2^{-*} released was determined by measuring the absorbency at 550 nm against blankappas containing fc and superoxide dismutase (SOD; 300 U/ml) [18, 19, 42]. All experiments were performed in duplicate and data are presented as the amount of O_2^{-*} released based on nmoles of reduced fc/min/mg protein.

Non-specific analysis of ROS. The relatively non-specific measure of ROS production is the reaction with dihydrorhodamine (DHR), thereby yielding water and a particularly stable tertiary free radical, which undergoes rearrangement of the pi electrons, leading to formation of fluorescent rhodamine [18, 19, 42, 43]. Cells were cultured at 10^5/well in flat-bottomed microtitre plates at normoxia. Wells were washed with sterile, pre-equilibrated saline, then cultures were continued in KAPPAreb's/Ringer solution containing 50 muM DHR and rhIL-1beta as indicated. Fluorescence was measured at excitation/emission wavelengths of 485/535 nm. Negative/positive controls were included to monitor the specificity of the reaction [18, 19]. ROS production by the control (no rhIL-1beta) was measured and calibrated to 100% and variables were plotted against this level as logarithmic fluorescence units.

*Pre-treatment with selective antioxidants prior to exposure to exogenous rhIL-1*beta

In order to investigate the hypothesis that ROS act as signaling messengers in rhIL-1beta-mediated regulation of HIF-1alpha, selective scavengers of O_2^{-*}, $^*OH$ and H_2O_2 were employed. Selection of scavengers was based on antioxidant potency and specificity: dimethyl sulfoxide (DMSO) is a prototypical scavenger of $^*OH$ [44], 1,3-dimethyl-2-thiourea (DMTU) scavenges both H_2O_2 and $^*OH$ [44-46] and 4'-hydroxy-3'-methoxy-acetophenone (HMAP) is an NADPH-oxidase inhibitor and may also blockappa ROS production by mitochondria [47]. Epithelial cells were pre-treated for 2 hours with DMSO (0, 0.04, 0.2, 1%), DMTU (0, 0.1, 1, 10 mM) or HMAP (0, 0.1, 1, 10 mM) before exposure to rhIL-1beta (10 ng/ml) for 2 hours. Nuclear extracts were prepared and DNA-binding activity of HIF-1alpha was performed. Separately, in order to determine the level of participation of mitochondrial ROS, cells were pre-treated with diphenylene iodonium (DPI; 0, 0.1, 1, 10 muM), a non-specific inhibitor of flavin-dependent O_2^{-*}-producing enzymes, including not only mitochondrial but also the classical NADPH oxidase [48, 49], or with KAPPACN (0, 0.1, 1, 10 mM), an inhibitor of the mitochondrial respiratory chain, followed by exposure to rhIL-1beta (10 ng/ml) for 2 hours and assessment of the nuclear translocation and DNA-binding activity of HIF-1alpha.

Statistical analysis and data handling

Data are presented as means ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by *post hoc* Tukappaey's test, and the *a priori* level of significance at 95% confidence level was considered to be p ¾ 0.05.

RESULTS

*The role of rhIL-1*beta *in regulating the stability and nuclear translocation of HIF-1*alpha

Figure 1A displays a gel of Western analysis for the effect of rhIL-1beta (10 ng/ml) on the nuclear localization and abundance of the HIF-1alpha subunit under normoxic conditions (± 21% O_2/5% CO_2). HIF-1alpha accumulation in the presence of rhIL-1beta increased significantly as early as 15 min after the addition of rhIL-1beta (Figure 1A). This nuclear accumulation of HIF-1alpha

increased exponentially (maximizing at 0.5-2 hours), remained elevated for ~ 48 hours and then declined to control (baseline) values between 72-96 hours (Figure 1A). The housekappaeeping protein, beta-actin, was used as an internal reference for semi-quantitative loading per lane (Figure 1A). Histogram analysis of the effect of rhIL-1beta on HIF-1alpha nuclear abundance is shown in Figure 1B.

*The effect of rhIL-1*beta *on the nuclear activation of HIF-1*alpha *as determined by phosphorimaging*

In association with its ability to up-regulate the nuclear translocation of HIF-1alpha, rhIL-1beta induced the DNA-binding activity of this transcription factor superimposed across the same time-response curve during normoxia (Figure 2A). EMSA analysis detected HIF-1alpha activity as early as 15 min after addition of rhIL-1beta (Figure 2A). It became maximally active at around 1-2 hours, remained elevated through ~ 16 hours and declined thereafter (Figure 2A). Histogram analysis of the gel-shifted bands as performed by phosphorimaging is displayed in Figure 2B. In order to verify that the shifted bands in response to rhIL-1beta treatment do in fact correspond to the HIF-1alpha subunit, supershift analysis was performed to determine their specificity. As shown in Figure 3, incubation of cells with rhIL-1beta (10 ng/ml) for 2 hours induced HIF-1alpha activity. Pre-incubating nuclear extracts treated with rhIL-1beta for 2 hours with a monoclonal antibody specific for the alpha subunit for 24 hours caused a supershift (SS; Figure 3). Nuclei pre-incubated for 24 hours with alpha-HIF-1b, the ubiquitously and constitutively expressed subunit of the HIF-1 complex, showed no supershifted band (Figure 3). To further delineate the specificity of HIF-1alpha bands, nuclear extracts were incubated in the presence of either the mutant oligonucleotide (M-18) or the wild type; M-18 suppressed the positive effect of rhIL-1beta on HIF-1alpha. Addition of a cold competitor abrogated rhIL-1beta-induced HIF-1alpha DNA binding activity in a dose-dependent manner (Figure 3), indicating that the gel-shifted band does represent HIF-1alpha.

*The excitatory role of rhIL-1*beta *in mediating the intracellular accumulation of ROS*

To further unravel the underlying mechanism of the mode of action of rhIL-1beta, the possible involvement of enhanced ROS production was investigated. As expected, rhIL-1beta induced, in a time-dependent manner, the accumulation of H_2O_2 (peakappa at ~ 1-2 hours) (Figure 4A), O_2^{-*} (peakappa at ~ 2 hours) (Figure

4B) and ROS (peakappa at ~ 1-2 hours), as determined by the fluorescence of rhodamine (Figure 4C).

*The effect of selective antioxidants on the stability and nuclear translocation of HIF-1*alpha

Figure 5 displays a gel of Western analysis for the effect of antioxidants on rhIL-1beta (10 ng/ml; 2 hours)-induced nuclear localization and abundance of the HIF-1alpha subunit under normoxic conditions ([a] 21% O_2/5% CO_2). HIF-1alpha accumulation in the presence of rhIL-1beta increased significantly at 2 hours, an effect attenuated in a dose-dependent manner by DMSO pre-treatment (Figure 5A). Similarly, DMTU reduced rhIL-1beta-induced nuclear localization and abundance of HIF-1alpha in a dose-dependent manner (Figure 5B). HMAP significantly reduced HIF-1alpha nuclear localization and abundance as shown in Figure 5C. In addition, DPI (Figure 5D) and KAPPACN (Figure 5E), mitochondrial inhibitors, reduced in a dose-dependent manner the nuclear localization and abundance of HIF-1alpha. The housekappaeeping protein, beta-actin, was used as an internal reference for semi-quantitative loading per lane.

*The effect of selective antioxidants on IL-1*beta*-induced HIF-1*alpha *activation*

To determine whether ROS production might be causally important, the role of selective antioxidants and the level of involvement of the mitochondrial complex in mediating the effect of rhIL-1beta on HIF-1alpha were investigated. The effect of DMSO, a potent scavenger of $^{*}OH$, on rhIL-1beta-dependent activation of HIF-1alpha (2 hours) is shown in Figure 6A, with percentile inhibition of 18.2 and 47.5 at 0.2 and 1%, respectively (IC_{50} = 1.02 ± 0.07%). The effect of DMTU, a prototypical and potent scavenger of $^{*}OH$, on rhIL-1beta-dependent activation of HIF-1alpha is shown in Figure 6B, with percentile inhibition of 15.2, 47.6 and 75.8 at 0.1, 1 and 10 mM, respectively (IC_{50} = 0.52 ± 0.07 mM). The effect of HMAP on rhIL-1beta-dependent activation of HIF-1alpha is shown in Figure 6C, with percentile inhibition of 25.2, 71.5 and 76.7 at 0.1, 1 and 10 mM, respectively (IC_{50} = 0.582 ± 0.10 mM). The effect of DPI on rhIL-1beta-dependent activation of HIF-1alpha is shown in Figure 6D, with percentile inhibition of 42.5, 59.7 and 78.5 at 0.1, 1 and 10 muM, respectively (IC_{50} = 0.62 ± 0.12 muM). The effect of KAPPACN, a prototypical inhibitor of the mitochondrial respiratory chain, on rhIL-1beta-dependent activation of HIF-1alpha is shown in Figure 6E, with percentile

inhibition of 27.5, 38.2 and 75.1 at 0.1, 1 and 10 mM, respectively (IC_{50} = 0.56 ± 0.10 mM).

DISCUSSION

The present investigation has revealed a novel role for ROS signaling in mediating a non-hypoxic effect of rhIL-1beta, an inflammatory cytokappaine, on HIF-1alpha stabilization, nuclear translocation and activation during normoxia. Despite the fact that HIF-1alpha was recognized as a transcriptional activator prevailing under hypoxic conditions [1-5], ROS signaling pathways that mediate the regulation of HIF-1alpha have emerged only recently [6, 7, 12, 20, 48-52]. Consistent with this notion, it is therein reported for the first time, a non-hypoxic pathway mediating the effect of rhIL-1beta in regulating the stabilization, translocation and activation of HIF-1alpha in a ROS-sensitive mechanism. These results suggest that hypoxia may not be the only major player in HIF-1alpha regulation and that this pathway mediated by rhIL-1beta, for the moment, specific to alveolar epithelial cells, may play a major role in controlling HIF-1alpha regulation in a non-hypoxic environment.

The concept therefore has been put forward that ROS and phosphorylation/dephosphorylation events are master regulators of HIF-1alpha induction and activation; however, the underlying pathways and potential signaling mediators likappaely to be implicated have yet to be identified. Regarding the mechanism of non-hypoxic, ROS-dependent regulation of HIF-1alpha, a major role for mitochondrial ROS generated at the complex III site was reported, thereby causing the accumulation of HIF-1alpha protein ostensibly responsible for the initiation of gene expression [47, 48]. Furthermore, depletion of the mitochondrial genome has been shown to reverse ROS-mediated induction of HIF-1alpha [47-49]. In addition, it has been reported that HIF-1alpha-dependent transcriptional activity in the induction of VEGF expression has defined a novel hypoxia-independent mechanism regulating vascular remodeling [52]. Further afield, recent evidence suggested that the reactive nitrogen species (RNS) pathway regulates the stability and activity of HIF-1alpha [50]. For instance, it was shown that the expression of nitric oxide (NO) synthase could cause HIF-1alpha accumulation, thus underscoring the role of NO as an intracellular activator of this transcription factor [51].

The molecular pathways defined by cytokappaines that potentially mediate HIF-1alpha regulation, are not well understood. Recently for instance, Hellwig-Bürgel and coworkappaers reported a novel role for IL-1beta and TNF-alpha as regulatory mediators in stimulating the DNA-binding activity of HIF-1alpha, coming to the conclusion that this transcription factor is likappaely involved in modulating gene expression during inflammation [12]. The augmented activity of HIF-1alpha due to IL-1beta was subsequently attributed, at least in part, to increased HIF-1alpha protein abundance under non-hypoxic conditions, whereas the effect of TNF-alpha was thought to be due to the concomitant activation of certain proteins that were proposed to be part of the activated complex [12]. Moreover, cAMP-responsive element binding protein (CREB) has been implicated in augmenting HIF-1alpha activity within its site in the EPO gene enhancer [53, 54]. Although no effect of cytokappaines on cAMP levels and protein kappainase A (PKAPPAA) activity was presented, it has been reported that cAMP analogues prevented the *in vitro* suppression of EPO production mediated by cytokappaines [53, 54].

Amongst pro-inflammatory cytokappaines, TNF-alpha and IL-1beta have perhaps been most exhaustively investigated [9-11, 14-28, 35-41]. For example, IL-1beta, likappae TNF-alpha, exerts its pleiotropic activities through ligand-induced cross-linkappaing of specific receptors [14, 36-40]. During inflammation, cytokappaines, including TNF-alpha and IL-1beta, transiently activate neutrophils and macrophages, thereby causing enhanced production of O_2^{-*}, possibly *via* the activation of NADPH oxidase [35-40]. This oxidative burst involves a rapid but transient release of ROS as a crucial part of the defense mechanisms against invading microbial pathogens and tumor cell metastasis. Although TNF-alpha and IL-1beta are primarily produced by macrophages, accumulating evidence suggests that other cell types such as endothelial and epithelial cells release IL-1beta, IL-6, TNF-alpha and other inflammatory mediators, thereby amplifying the inflammatory responses by activating and recruiting inflammatory cells [18-28]. In this respect, it has been reported that cytokappaines up-regulate intracellular accumulation of ROS [11, 12, 18-20, 32, 37, 55, 56]. More recently, studies by Corda *et al.* and Esposti and coworkappaers indicated that cytokappaines increase mitochondrial ROS generation, suggesting that their effect could be mediated through a ROS-sensitive mechanism [57, 58]. The results therein reported indicate a potential role for IL-1beta in inducing the accumulation of intracellular ROS, thereby reinforcing the notion that this cytokappaine mediates its effect on transcription factors

including HIF-1alpha through a ROS-dependent mechanism. Regarding the likappaely source of ROS production engendered by rhIL-1beta, at least two sources may be involved: i) the membrane-bound NADPH oxidase and ii) the mitochondrial respiratory chain. Although ROS, in general, may be involved in mediating the effect of rhIL-1beta on HIF-1alpha induction, the latter mechanism probably predominates because blockappaade of mitochondrial respiration abrogates rhIL-1beta-dependent activation of HIF-1alpha. Furthermore, evidence is provided that different ROS species (O_2^{-*}, $^*OH$ and H_2O_2) may mediate the effect of rhIL-1beta on HIF-1alpha stabilization, localization and activation. The observation that a non-hypoxic pathway mediates the effect of rhIL-1beta on HIF-1alpha involves ROS generated within the mitochondrial complex is supported by equivocal evidence since DPI, an inhibitor of complex I nicotinamide adenine dinucleotide phosphate-dependent oxidase (which blockappaades the conversion of ubiquinone = › ubiquinol), abrogates rhIL-1beta-mediated activation of HIF-1alpha, indicating a crucial role for mitochondria-derived ROS in HIF-1alpha signaling. Although it has been suggested that ROS mediate their signaling by affecting kappainases and/or phosphatase activities [2-4, 6, 7, 14, 18, 19, 37, 38], the precise downstream pathway(s) affected by ROS, which govern HIF-1alpha translocation/activation, have yet to be identified. A simulated schematic overview summarizing the potential signaling pathways mediating cytokappaine-dependent regulation of HIF-1alpha is shown in Figure 7.

The present investigation has, however, thrown some light on the mechanism whereby rhIL-1beta promotes HIF-1alpha activity. The results are highlighted as follows: i) exposure to rhIL-1beta induces the non-hypoxic stabilization and nuclear translocation of HIF-1alpha; ii) this effect is associated with the up-regulation of HIF-1alpha activity during normoxia; iii) rhIL-1beta augments the intracellular accumulation of various ROS radicals; iv) selective antioxidants, purported to specifically scavenge and neutralize free radicals, attenuate rhIL-1beta-mediated activation of HIF-1alpha; and v) blockappaading the mitochondrial complex I and the dynamic flow of its respiratory chain electrons abrogates rhIL-1beta-dependent regulation of HIF-1alpha. It is concluded that the production of ROS is clearly involved in rhIL-1beta-mediated normoxic regulation of HIF-1alpha stabilization, translocation and activation.

CONCLUSION

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Figures

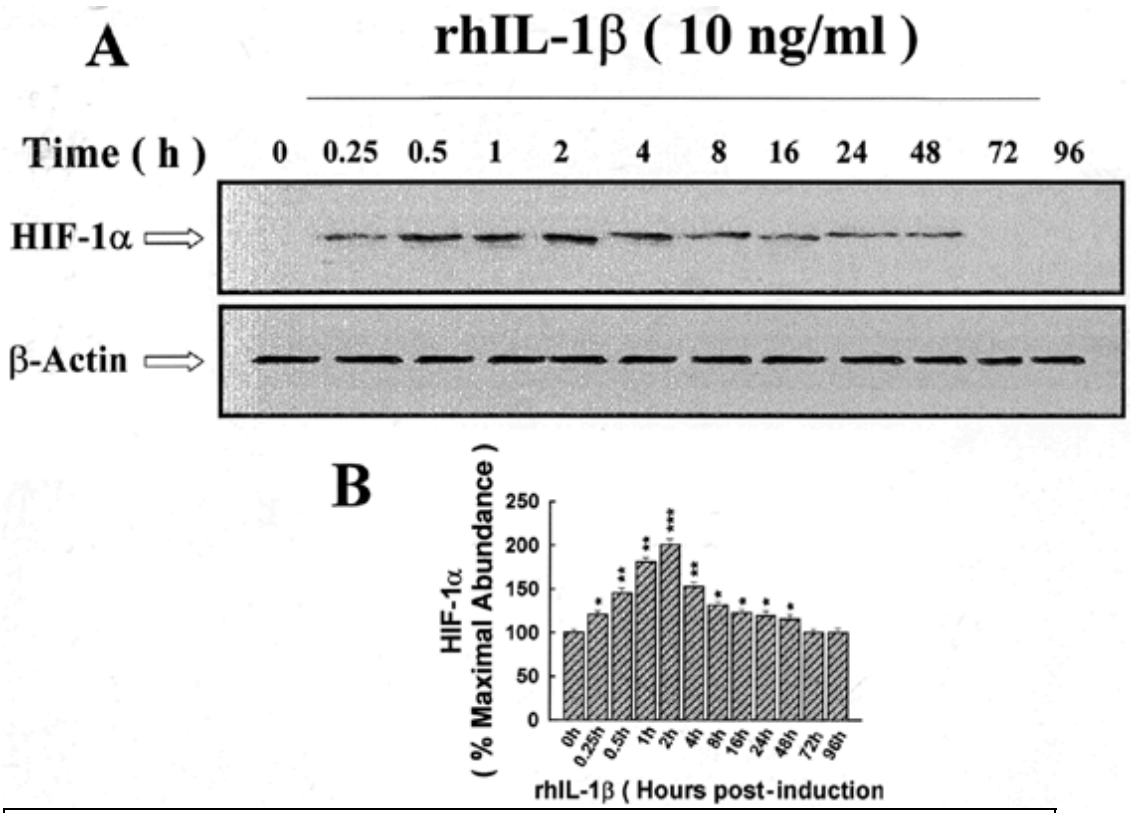


Figure 1

Western analysis of rhIL-1beta-mediated regulation of HIF-1alpha stabilization and translocation.

A: Incubation of epithelial cells under normoxic conditions with rhIL-1beta (10 ng/ml), induced the stabilization and

nuclear accumulation of HIF-1alpha, in a time-dependent manner. HIF-1alpha was detected as early as 15 minutes post-addition of rhIL-1beta, maximized at around ~ 1-2 hours, remained relatively abundant at 24-48 hours and declined thereafter. beta-Actin was used as an internal reference for the semi-quantitative loading for each lane. **B:** Histogram analysis of the effect of rhIL-1beta on HIF-1alpha nuclear abundance. The nuclear abundance of HIF-1alpha was determined relative to the control in the absence of rhIL-1beta. Maximum abundance was observed at ~ 2 hours, thereafter declining. Results are representative of three independent experiments.

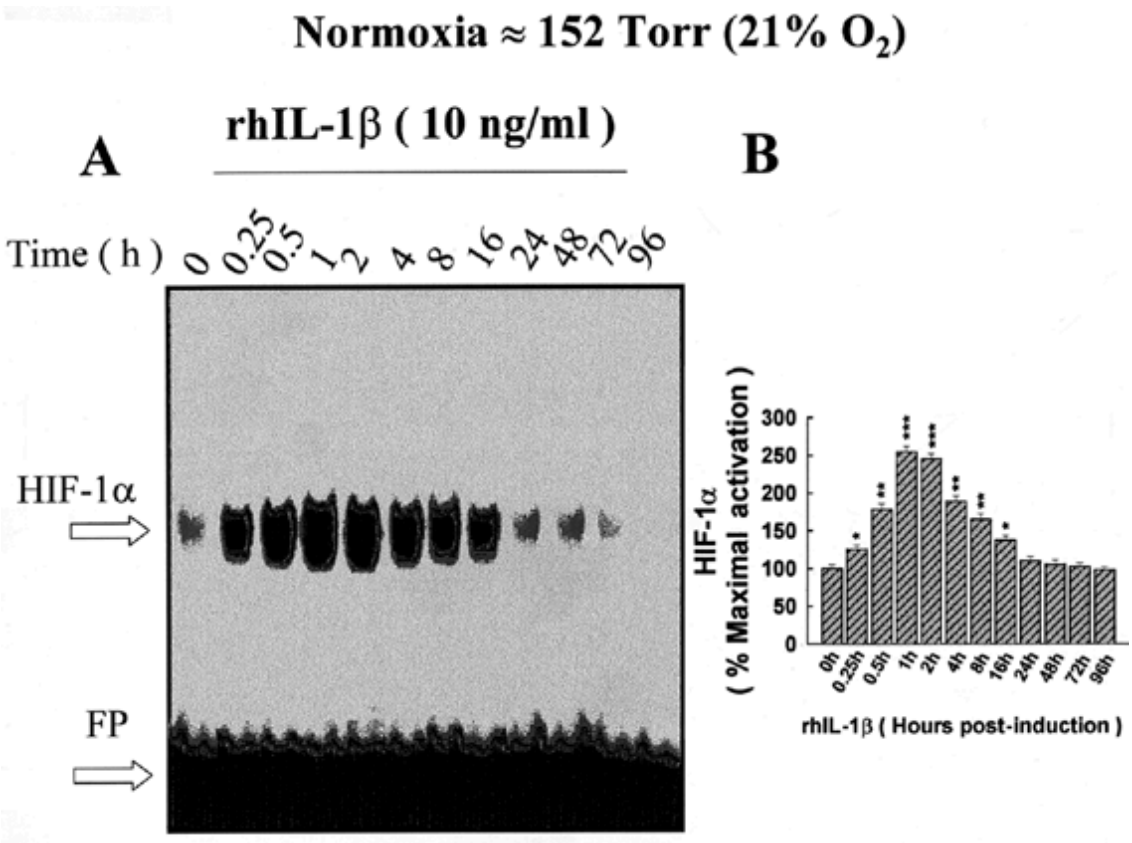


Figure 2

The effect of rhIL-1beta on HIF-1alpha activation under atmospheric oxygen tension.

A: In association with causing the nuclear translocation of HIF-1alpha, rhIL-1beta increased the DNA-binding activity in a time-dependent manner. HIF-1alpha activity was detected as early as 15 minutes post-addition of rhIL-1beta, maximized at around 1-2 hours and remained elevated for 16 hours. The faster migrating probe (FP) indicates the free, unbound probe and the upper band is that of the HIF-1alpha/DNA complex. **B:** Histogram analysis of the gel-shifted bands as determined by phosphorimaging. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as compared with control in the absence of rhIL-1beta. Results are representative of three independent experiments.

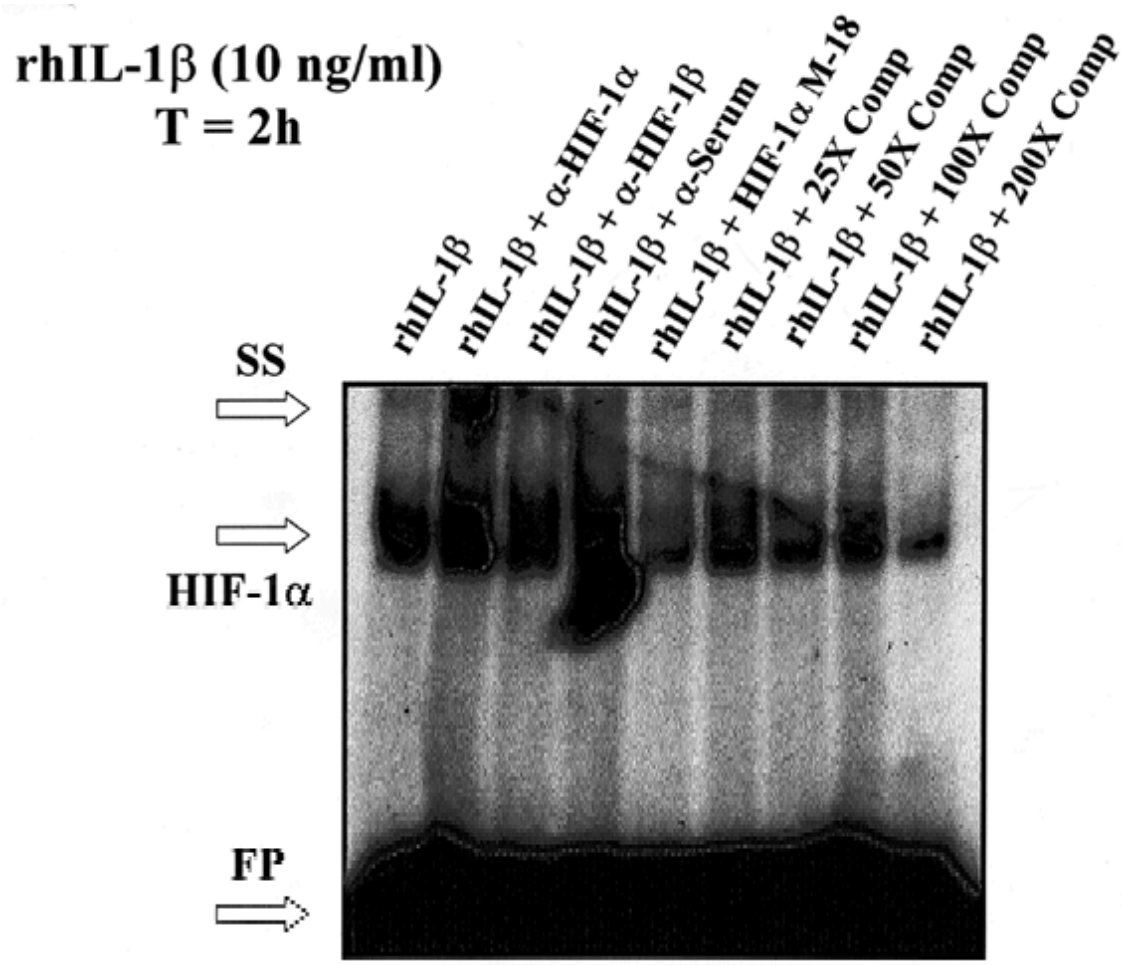


Figure 3

Specificity of HIF-1alpha gel-shifted bands in response to rhIL-1beta (10 ng/ml; 2 hours) treatment. Addition of rhIL-1beta induced the activity of HIF-1alpha.

Pre-incubating (for 24 hours) nuclear extracts exposed to rhIL-1beta with a selective alpha-HIF-1alpha monoclonal antibody, prior to the addition of wild type oligonucleotide, caused the appearance of a further retarded band (supershift, SS). Pre-incubating (for 24 hours) nuclear extracts exposed to rhIL-1beta with a selective alpha-HIF-1beta monoclonal antibody, prior to the addition of wild type oligonucleotide, did not cause the appearance of a further retarded band, thereby indicating the presence of a viable alpha subunit. Pre-incubating (for 24 hours) nuclear extracts exposed to rhIL-1beta with a non-selective serum, prior to the addition of wild type oligonucleotide, did not cause the appearance of a further retarded band, indicating specificity. The mutant oligonucleotide (HIF-1alpha M-18) abolished the appearance of the shifted band. To confirm the specificity determined by the supershift, cold oligonucleotide competitor was added with the wild type. The cold competitor (COMP) abolished, dose-dependently, the specific band of HIF-1alpha obtained due to rhIL-1beta. The faster migrating probe (FP) indicates the free, unbound probe, the upper shifted band that of the HIF-1alpha/DNA complex, and SS indicates the super shift. Results are representative of three independent experiments.

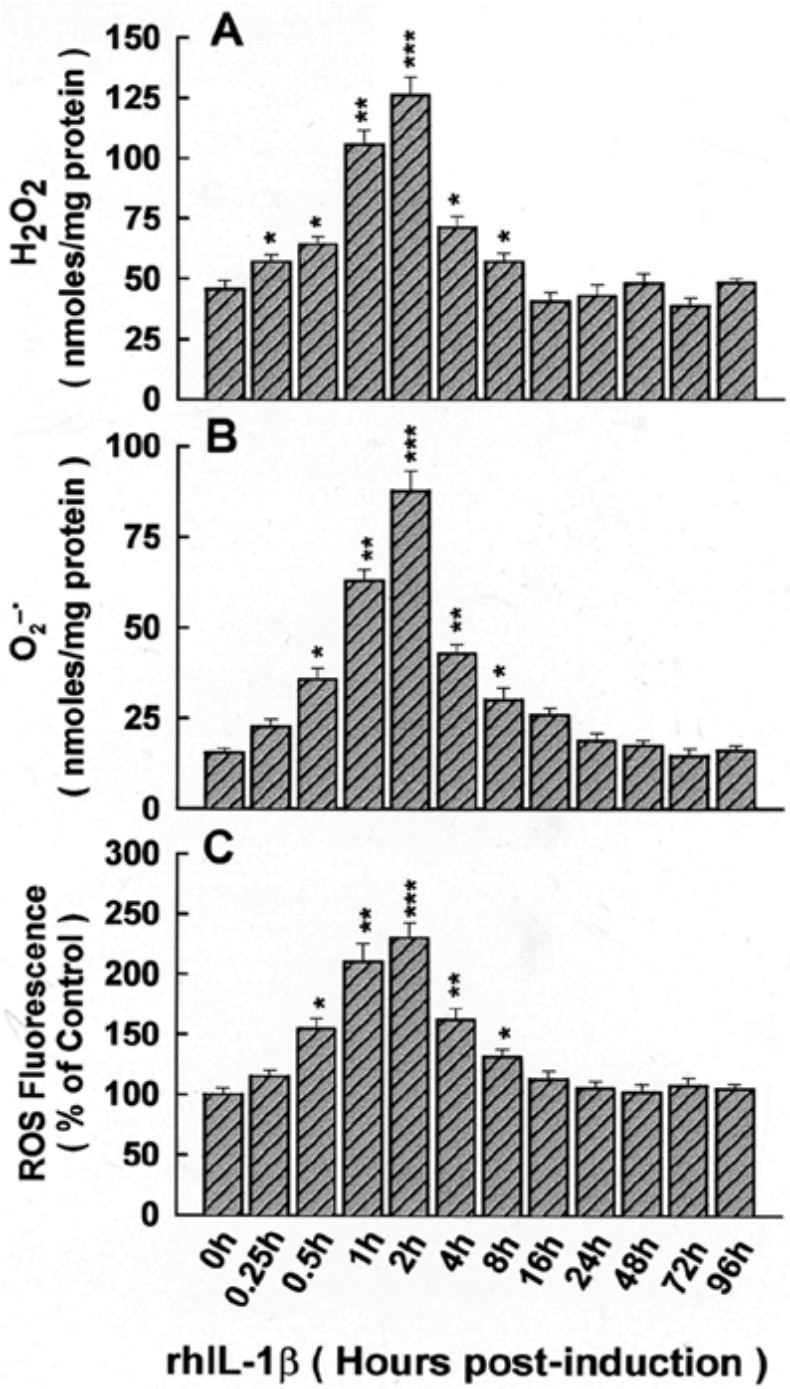


Figure 4

Analysis of reactive oxygen species (ROS) generated in response to rhIL-1beta (10 ng/ml) treatment.

A: The effect of rhIL-1beta on the accumulation of hydrogen peroxide (H_2O_2). The concentration of H_2O_2 began to increase 15 min after addition of rhIL-1beta, maximized at 1-2 hours and continued to be significantly higher than the control up to 8 hours, thereafter

declining. **B:** The effect of rhIL-1beta on the accumulation of superoxide anion (O_2^{-*}). The concentration of O_2^{-*} began to increase 30 min after addition of rhIL-1beta, maximized at 2 hours and continued to be significantly higher than the control until 8 hours. **C:** The effect of rhIL-1beta on the non-specific accumulation of ROS. ROS (fluorescence) began to increase 30 min after addition of rhIL-1beta, maximized at 1-2 hours and continued to be significantly higher than the control until 8 hours. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as compared with the control in the absence of rhIL-1beta. Results are representative of four independent experiments run in duplicate.

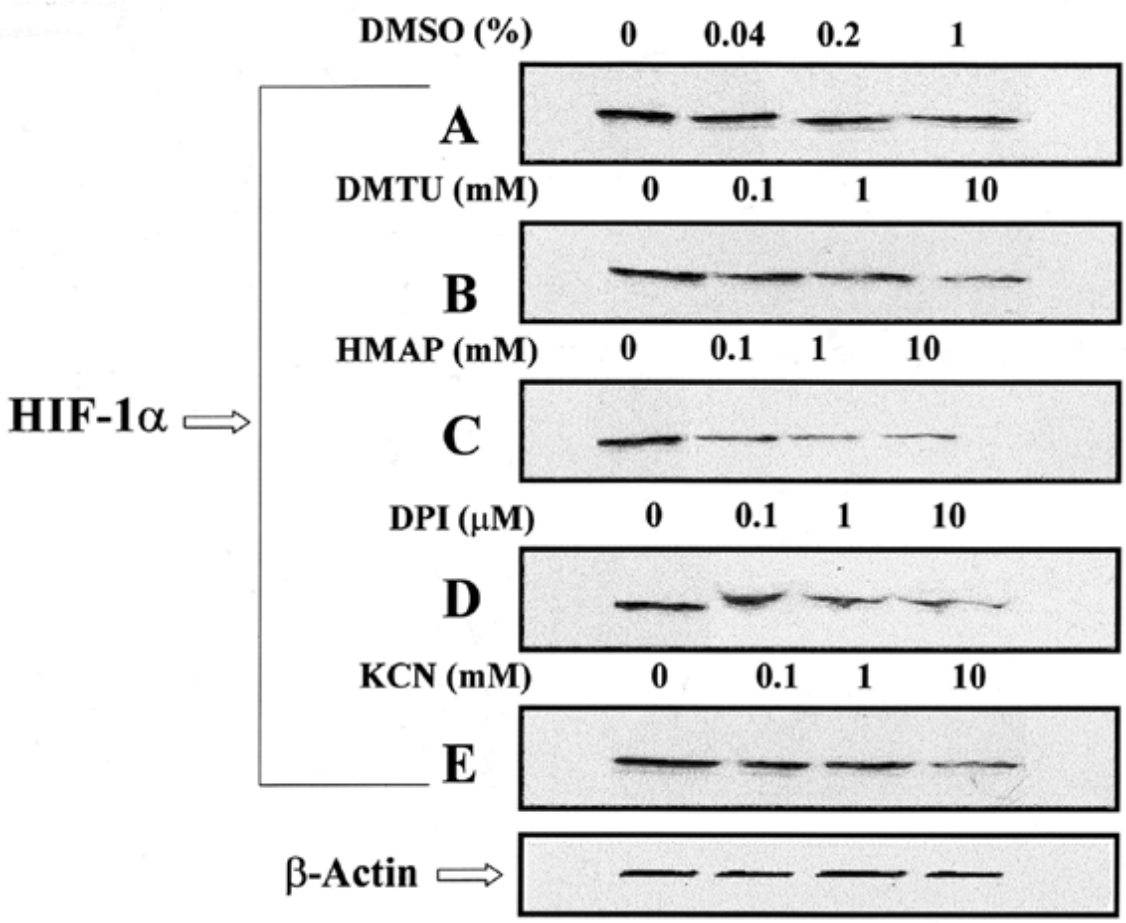


Figure 5

Analysis of the effect of selective antioxidants and mitochondrial respiration inhibitors on rhIL-1beta (10 ng/ml; 2 hours) mediated nuclear translocation of HIF-1alpha.

A: Dose-dependent inhibitory effect of DMSO on HIF-1alpha abundance. **B:** Dose-dependent inhibitory effect of DMTU on HIF-1alpha abundance. **C:** Dose-dependent inhibitory effect of HMAP on HIF-1alpha abundance. **D:** Dose-dependent inhibitory effect of DPI on HIF-1alpha abundance. **F:** Dose-dependent inhibitory effect of KAPPACN on HIF-1alpha abundance. beta-Actin was used as an internal reference for semi-quantitative loading for each lane. Results are representative of three independent experiments.

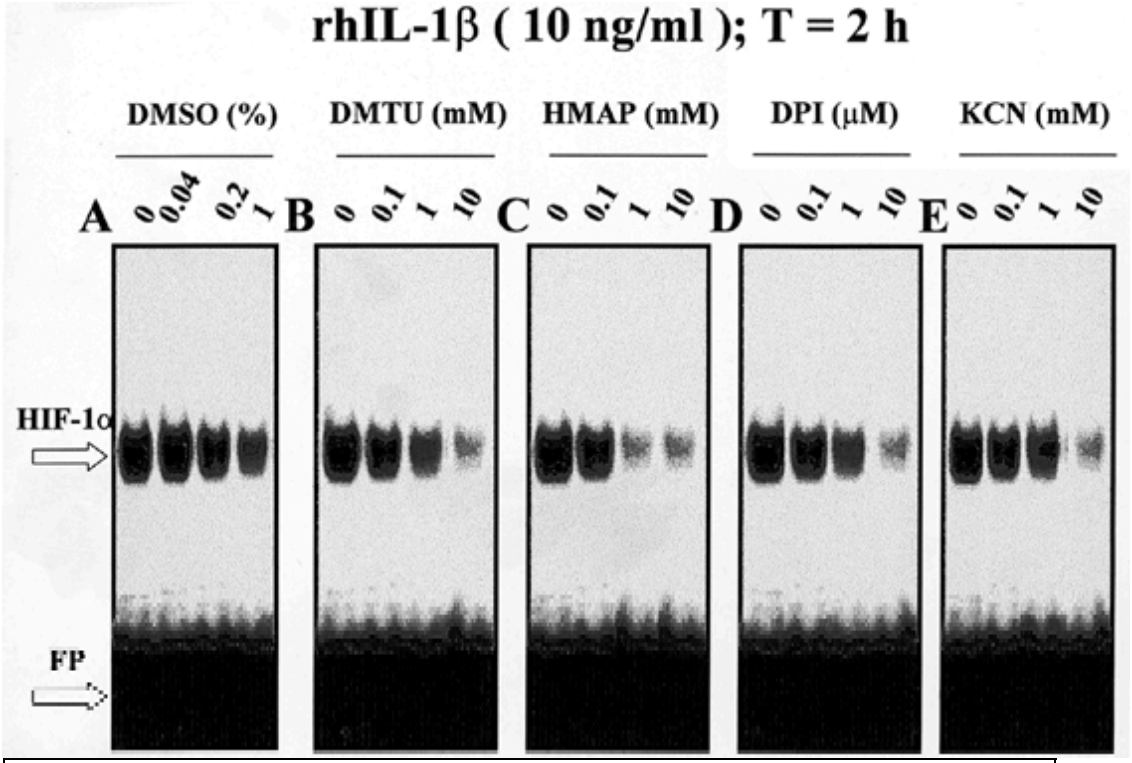


Figure 6

Analysis of the effect of selective antioxidants and mitochondrial respiration inhibitors on rhIL-1beta (10

ng/ml; 2 hours) mediated activation of HIF-1alpha.

A: Dose-dependent inhibitory effect of DMSO on HIF-1alpha activation. **B:** Dose-dependent inhibitory effect of DMTU on HIF-1alpha activation. **C:** Dose-dependent inhibitory effect of HMAP on HIF-1alpha activation. **D:** Dose-dependent inhibitory effect of DPI on HIF-1alpha activation. **F:** Dose-dependent inhibitory effect of KAPPACN on HIF-1alpha activation. Results are representative of three independent experiments.

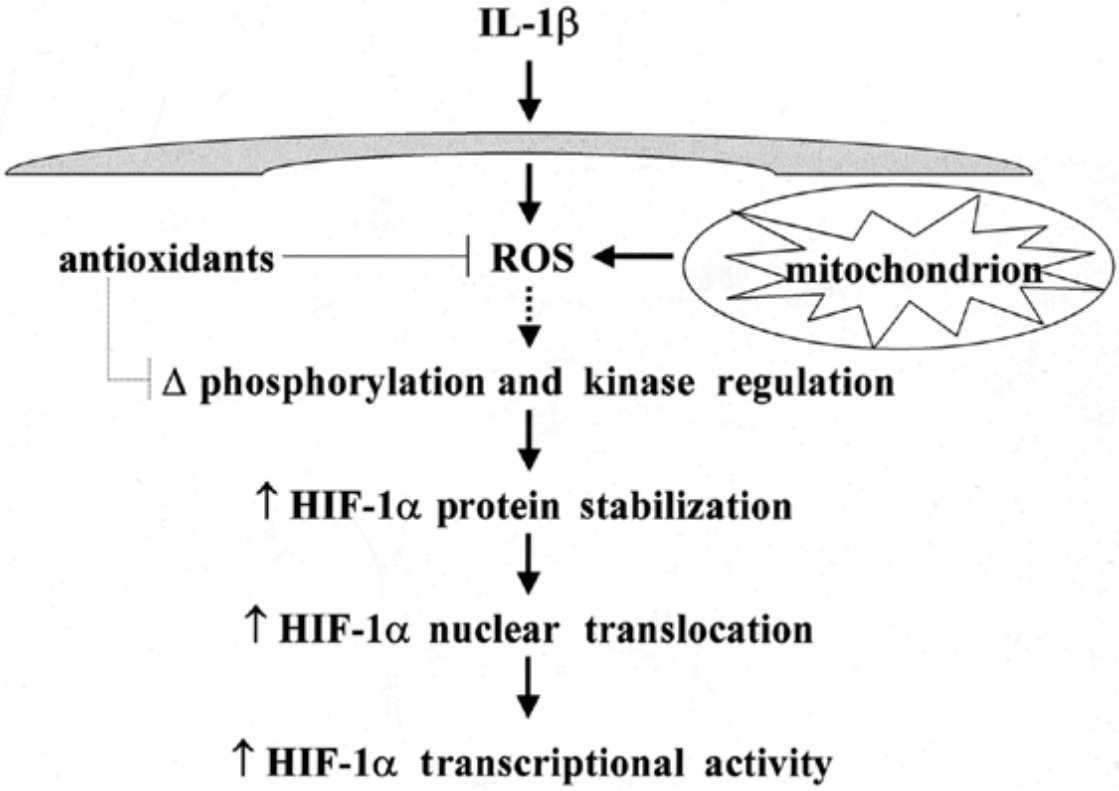


Figure 7

A schematic overview of the potential signaling pathways involved in rhIL-1beta-mediated regulation of HIF-1alpha during normoxia.

rhIL-1beta induces the intracellular accumulation of ROS, which may cause changes in the phosphorylation state of

target kappainases, thereby mediating specific regulatory mechanism. The mitochondrion is a potential source of rhIL-1beta-unleashed ROS, the regulation of which is selectively mediated by antioxidants. ROS-mediated signaling allows HIF-1alpha protein stabilization, nuclear translocation and transcriptional activation.

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In this issue

Osteoprotegerin and inflammation

N. Saidenberg Kermanac'h, Natacha Bessis, M. Cohen-Solal, M.C. De Vernejoul, Marie-Christophe Boissier
bone resorption, inflammation, osteoprotegerin, RANK.

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A.-L. Chamorey, N. Magné, X. Pivot, G. Milano
cytokine glycosylation, oncology, erythropoietin, granulocyte-colony stimulating factors, interleukins, interferons.

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M. G. Smirnova, S. L. Kiselev, N. V. Gnuchev, J. P. Birchall, J. P. Pearson
endotoxin, cytokines, middle ear inflammation, otitis media.

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M. H. Deininger, P. G. Kremsner, R. Meyermann, H. Schluesener
cerebral malaria, cytokines, macrophages.

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dendritic cells, cytokines, generation, maturation, differentiation.

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interleukin-10, polymorphism, microsatellite, multiple sclerosis.

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G. Vento, P. G. Matassa, F. Ameglio, E. Capoluongo, L. Tortorolo, C. Romagnoli
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S. Kaser, A. Kaser, W. Vogel, J. R. Patsch, H. Tilg
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The CD14-260 C => T promoter polymorphism co-segregates with the tumor necrosis factor-alpha (TNF-alpha)-308 G => A polymorphism and is associated with the

interleukin-1beta (IL-1beta) synthesis capacity of human leukocytes

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CD14, tumor necrosis factor-alpha, interleukin-1beta, endotoxin, genetic polymorphism, linkage disequilibrium, polymerase chain reaction.

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breast cancer, cell line, extracellular matrix (ECM), tumor cell phenotype, malignant potential, cytokine expression pattern.

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nicotine, macrophage inflammatory protein-1alpha (MIP-1alpha), rat alveolar macrophage (RAM).

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