Microtubule Disruption Utilizes an NFκB-dependent Pathway to Stabilize HIF-1α Protein*

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Received for publication, September 24, 2002, and in revised form, December 11, 2002
Published, JBC Papers in Press, December 17, 2002, DOI 10.1074/jbc.M209804200

Hypoxia-inducible factor (HIF)-1α levels are elevated in normoxic cells undergoing physiological processes involving large scale microtubule reorganization, such as embryonic development, wound healing, and tumor cell metastasis. Although alterations in microtubules affect numerous cellular responses, no data have yet implicated microtubule dynamics in HIF-1α regulation. To investigate the effect of microtubule change upon HIF-1α regulation, we treated cells with the microtubule-depolymerizing agents (MDAs) colchicine, vinblastine or nocodazole. We demonstrate that these agents are able to induce transcriptionally active HIF-1. MDA-mediated induction of HIF-1α required microtubule depolymerization, since HIF-1α levels were unchanged in cells treated with either the microtubule-stabilizing agent paclitaxel, or an inactive form of colchicine, or in colchicine-resistant cells. HIF-1 induction was dependent upon cellular transcription, as transcription inhibitors abrogated HIF-1α protein up-regulation. The ability of transcriptional inhibitors to interfere with HIF-1α accumulation was specific to the MDA-initiated pathway, as they were ineffective in preventing hypoxia-mediated HIF-1 induction, which occurs by a distinct post-translational pathway. Moreover, we provide evidence implicating a requirement for NFκB transcription in the HIF-1 induction mediated by MDAs. The ability of MDAs to induce HIF-1α is dependent upon activation of NFκB, since inhibition of NFκB either pharmacologically or by transfection of an NFκB super-repressor plasmid abrogated this induction. Collectively, these data support a model in which NFκB is a focal point for the convergence of MDA-mediated signaling events leading to HIF-1 induction, thus revealing a novel aspect of NFκB regulation and function.

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‡ The abbreviations used are: HIF-1, hypoxia inducible factor-1; MDA, microtubule-depolymerizing agent; vin, vinblastine; col, colchicine; noco, nocodazole; taxol, paclitaxel; ActD, actinomycin-D; DCB, dichlorobenzimidazole riboside; phe, phenanthroline; γ-lumicolchicine; NFκB, nuclear factor κB; NFRP, NFκB super-repressor plasmid; iNOS, inducible nitric-oxide synthetase; ARNT, aryl hydrocarbon nuclear translocator; HA, hemagglutinin; PDTC, pyrrolidine dithiocarbamate; CHX, cycloheximide; PI, phosphatidylinositot; CMV, cytomegalovirus.

A nuclear translocator (ARNNT, HIF-1β) (1). HIF-1α and HIF-1β mRNAs are constantly expressed under normoxic and hypoxic conditions (2). However, HIF-1α protein is significantly increased by hypoxia, whereas the HIF-1β protein remains constant regardless of oxygen tension (3). Under normoxia, HIF-1α protein is remarkably unstable and its degradation by the proteasome is orchestrated by the ubiquitin protein ligase VHL (3–6). Under normoxia, VHL recognizes HIF-1α as a substrate due to the enzymatic modification of HIF-1α by prolyl hydroxylases, whose function is inhibited during hypoxia (7, 8). Hypoxic stabilization of HIF-1α is accompanied by its nuclear translocation, heterodimerization with HIF-1β, and transcription of genes encoding proteins that function to increase O2 delivery, allow metabolic adaptation, and promote cell survival (9). HIF-activated genes such as iNOS, IGF, and VEGF play an important role in tumor metastasis and invasion (10, 11) and HIF-1α protein is overexpressed in a majority of non-hypoxic metastatic tumors and cell lines (12).

One of the major components of the cytoskeleton is the microtubule network. Because of the dynamic instability of tubulin dimers, microtubules are subject to constant remodeling (13). MDAs are potent anti-tumor agents that associate with microtubules and disrupt the microtubular system, thereby blocking cell division (14–16). The action of MDAs is thought to loosely mimic a wide range of cellular responses involving cytoskeletal rearrangement, such as wound healing, tumor cell metastasis, and invasion (17, 18). Microtubule reorganization has been also shown to correlate with changes in gene expression (19–21). For instance, it has been reported that microtubule disruption by MDAs modulates gene expression and activity of protein kinases and transcription factors such as NFκB (22–28).

NFκB is an ubiquitous transcription factor known to be activated by a wide variety of stimuli including infection, inflammation, oxidative stress, and the aforementioned microtubule disruption (29). NFκB transactivates a number of proinflammatory, apoptotic and oncogenic genes that collectively function to foster cellular adaptation to stress (29, 30). Although the mechanism of activation depends on the stimulus, most stimuli initiate various intracellular signaling cascades that result in the phosphorylation of inhibitory protein κB (IκB) by IκB kinases (IKKs) (31). NFκB is normally associated with IκB in the cytoplasm, where it is kept in an inactive state (32). Stimulus-mediated phosphorylation and subsequent proteolytic degradation of IκB (33, 34) allows the release and nuclear translocation of NFκB, where it transactivates a number of target genes.

The pathways involved in the nonhypoxic stabilization of HIF-1α remain unclear but are thought to be regulated by growth factor signaling cascades such as PI 3-kinase/AKT (35, 36). Interestingly, HIF-1α has been reported to be highly ex-
pressed in cells during physiological processes that entail mas-
sive microtubule reorganization (12, 37–39). However, there are
no reports demonstrating a direct relationship between changes in
microtubule dynamics and HIF-1α protein regulation. Therefore, in
this study, we specifically investigated this connection. We show that reagents interfering with tubulin polymerization are able to induce NFκB transcription and we further show that this activation is necessary for the sub-
sequent increase in HIF-1α protein expression. These results
demonstrate a novel aspect of HIF-1α regulation and suggest that
HIF-1α may play a broader role in sensing cytoskeletal
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MATERIALS AND METHODS

Reagents—The MDAs vinblastine, colchicine, and nocodazole, and
the microtubule stabilizing agent paclitaxel were purchased from
Sigma. The colchicine derivative γ-lumicolchicine and pyrroldimedi-
thio carbamate and the transcriptional inhibitors actinomycin D (AcD)
and 5,6-dichlorobenzimidazole riboside were also purchased from
Sigma. Cobalt chloride and the iron chelator phenanthroline were
obtained from the same supplier. The protein synthesis inhibitor cyclo-
heximide (CHX) was from Sigma.

Cells and Transient Transfection—A549 cells (human lung cancer
cell line obtained from American Type Culture Collection) were cul-
tured in F12-K (Kaighn’s modification) medium (Invitrogen). Colch-
icine-resistant CHO (10193) and wild type CHO (10001) (a gift from Dr.
M. M. Gottesman, National Institutes of Health) were grown in α-mod-
ified minimal essential medium (Sigma). Jurkat cells were cultured in
RPMI 1640 medium (Biofluids). Unless specified, all other cell lines
were grown in Dulbecco’s modified Eagle’s medium (DMEM, Biofluids).
Media were supplemented with 10% fetal bovine serum, glutamine (for
DMEM), Hepes, and penicillin/streptomycin. For transient transfection
of NFκB super-repressor plasmid (40) or HIF-1α plasmids (41), cells
were plated in 6-cm dishes and transfected with NFκB super-
repressor plasmid (5 ug) or HIF-1α plasmids (3 ug) in the presence of
FuGENE 6 (Roche Molecular Biochemicals). After 24 h, cells were
subjected to the indicated drug treatments, lysates were harvested, and
HIF-1α levels determined by Western blotting. HIF-1 protein stability
was determined by treatment of cells with 200 μM CHX, followed by
immunoblot and densitometric analysis. For transient transfection
of reporter plasmids, cells were plated in 12-well plates and the following
day, cells were co-transfected with luciferase reporter plasmids contain-
ing either HIF-1α or NFκB binding sites (0.4 μg, a gift from Dr. M. Birrer, NCI) or
3X hypoxia response element (0.4 μg, a gift from Dr. G. Melillo, NCI), in
combination with the internal control CMV Renilla luciferase plas-
mid (1:100 the amount of reporter plasmid, Promega). Luciferase activ-
ies of reporter plasmids were measured using the Dual-Lucase
Reporter Assay System (Promega). Transfection efficiency was evalu-
ated using green fluorescence protein expression and determined to
be 35–45% under these experimental conditions. Cell viability
was determined by the trypan blue exclusion method. Cell viability
was unchanged in each experimental condition.

Western Blotting—Cells were lysed and nuclear and cytosolic ex-
tracts prepared as described (42). Cell lysates were electrophoretically
separated using either 4–20 or 7.5% SDS-PAGE gels (BioRad). Proteins
were transferred to nitrocellulose membrane (Protran, Schleicher &
Schuell) and immunoblotted with either monoclonal anti-HIF-1α, poly-
clonal anti-iNOS antibodies (1:300 and 1:500, respectively, Transduc-
tion Laboratories), monoclonal anti-HA antibody (1:1000, Covance) or
monoclonal anti-β-tubulin antibody (1:2000, Santa Cruz Biotechnol-
y). HIF-1α in human cell lines was detected in 20 μg of nuclear extracts
and HIF-1α in non-human cell lines was detected in 30–40 μg of
nuclear extracts using monoclonal HIF-1α antibody (1:750, Novus).
iNOS protein was detected in 40 μg of cytosolic extracts. All blots
were developed with SuperSignal chemiluminescence substrate (Pierce) us-
ing chemiluminescent mouse horseradish peroxidase IgG (Amersham
Biotechnologies).

Quantitative RT-PCR Analysis for HIF-1α Expression—Cells were
treated with MDAs for 3 h and lysed and total mRNA was extracted
using RNeasy Mini Kit (Qiagen). The real-time quantification of HIF-1α
mRNA was carried out using SYBR Green PCR Master Mix (Applied Biosystems)
with the following primer pairs: human HIF-1α forward, 5′-TCCATG
TAGTCTTCCCTGATCA-3′, human HIF-1α reverse, 5′-TTTGAAGCCTTG
TCGCCCTTCA-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 ng each primer, 1× SYBR Green PCR Master Mix
(Applied Biosystems) containing a reference dye, and cDNA at 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

MDAs Induce HIF-1α in Various Cell Lines under Normoxia—Overexpression of HIF-1α and large scale changes in
microtubule organization are common events associated with
embryonic development, wound healing, and tumor cell inva-
sion and metastasis. We therefore sought to investigate
whether a correlation existed between microtubule disruption
and HIF-1α expression. To examine this question, A549 cells
were treated with the MDAs vinblastine, colchicine, or nocoda-
zole, or with the microtubule-stabilizing agent paclitaxel, and
HIF-1α protein expression was monitored. The concentrations
used represent those required for maximal microtubule disrupt-
ion (43). As shown in Fig. 1A, HIF-1α protein levels were
similarly induced by all of the MDAs tested, while HIF-1α
protein remained unchanged following treatment with pacli-
taxel, indicating that increased HIF-1α protein expression corre-
lated with microtubule depolymerization, and not with sta-
bilization. In Fig. 1B, the kinetics of vinblastine-mediated
HIF-1α induction were investigated. The data indicate that
the increase in HIF-1 levels is somewhat transient, with maximal
induction occurring between 4–5 h and rapidly declining by 7 h.

To determine whether the effect of MDAs on HIF-1α expres-
sion was a general phenomenon, we assessed the ability of
MDAs to induce HIF-1α levels in a variety of cell lines. As
shown in Fig. 1C, MDAs induced HIF-1α protein in cells de-
derived from multiple lineages, irrespective of tumorigenicity or
cell adherence, thereby demonstrating that this is a general
signaling pathway shared by many, if not all, cell types.

MDAs Induce Both NFκB-dependent Transcription and Up-
regulation of HIF-1α Protein—We previously found that the
inflammatory cytokines TNF-α and IL-1β induce HIF-1α pro-
tein expression via NFκB activation.2 Coincidently, MDAs are
reported to activate NFκB gene transcription (24). There-
fore, we wished to determine whether the HIF-1 induction
following MDA treatment was potentially mediated by an NFκB-
dependent pathway. First, we tested whether MDAs were ca-
ble of inducing NFκB activation in A549 cells, as assessed
with transiently transfected NFκB-responsive luciferase con-
structs. As shown in Fig. 2A, the MDAs, but not paclitaxel,
which promotes microtubule polymerization and stabilization,
induced NFκB-responsive luciferase activity. Interestingly,
MDA-induced NFκB activity correlated with the ability of these
agents to induce HIF-1α protein levels (Fig. 1). We therefore
explored the apparent correlation between MDA-induced
NFκB activation and HIF-1α protein induction. To investigate
this association, transiently transfected A549 cells were
treated with increasing concentrations of either vinblastine or
colchicine and NFκB activity was measured in parallel with
HIF-1α protein expression. As shown in Fig. 2B, treatment of
A549 cells with these agents resulted in a maximal level of
NFκB activity, followed by a decline in activity at higher con-
centrations. When HIF-1α protein levels were examined from
identically treated cells, the MDA-dependent increase in NFκB
activity mirrored the increase in HIF-1α levels and maximal
HIF-1α expression correlated with maximal NFκB activity.
Similarly, at higher doses, HIF-1α protein levels declined in
parallel with decreasing NFκB activity.

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submitted manuscript.
**MDA-mediated HIF-1 Induction**

**MDA-dependent HIF-1 Induction Is Dependent upon New Transcription and NFκB Activation**—We sought to determine whether the NFκB activation induced by MDAs was responsible for mediating the increase in HIF-1α protein. First, we examined the requirement for general transcription in the MDA pathway. Cells were treated with MDAs in the presence of the transcription inhibitors AcD or 5,6-dichlorobenzimidazole riboside (DCB) and HIF-1α levels were examined. As shown in Fig. 3A (upper panel), HIF-1 induction by MDAs was completely inhibited by either of these agents. To confirm that the effect of transcriptional inhibitors upon HIF-1α levels was specific for the MDA-mediated pathway, these agents were added in combination with hypoxia mimetics. Cobalt chloride and phenanthroline (an iron chelator) are known to stabilize HIF-1α by preventing prolyl hydroxylases from modifying the protein, thereby rescuing HIF-1α from the destabilizing effects of VHL. As shown in Fig. 3A (lower panel), the transcription inhibitors did not interfere with the ability of hypoxia mimetics to induce HIF-1α protein. This result demonstrates a requirement for cellular transcription in MDA-mediated HIF-1 induction, thus defining this pathway as distinct from the hypoxia-mediated pathway.

We next examined whether the NFκB pathway was specifically implicated in the MDA-mediated induction of HIF-1α. To test this, A549 cells were transiently transfected with an NFκB super-repressor plasmid (expressing IkB mutated to resist proteasome-mediated degradation) that effectively inhibits NFκB transcription (40), and these cells were subsequently subjected to treatment with MDAs. As shown in Fig. 3B (upper panel) MDA-mediated HIF-1 induction is severely impaired in the presence of NFκB repressor, thereby demonstrating a requirement for NFκB transcription in this pathway. To ensure that this result was not a nonspecific effect related to the transfections, the NFκB-inhibiting drug pyrrolidine dithiocarbamate (PDTC) was used to confirm these observations. As shown in Fig. 3B (lower panel), similar to the NFκB repressor effects, PDTC abrogated MDA-mediated HIF-1 induction. However, it had no effect upon the ability of hypoxia mimetics to induce HIF-1α, thereby validating the specificity of this pathway and emphasizing the crucial role of NFκB. To examine the effect of transcriptional inhibition upon MDA-stabilized HIF-1α protein, A549 cells were pretreated with vinblastine for 3 h, followed by either a 1 or 2 h treatment of actinomycin D (in the continued presence of vinblastine). As shown in Fig. 3C, transcription inhibition was able to moderately reduce, but not eliminate, already stabilized HIF-1 protein. These data demonstrate that while constitutive transcription is needed for MDA-mediated up-regulation of HIF-1α, persistence of the stabilized protein is not dependent on transcription.

In addition to the NFκB pathway, it has been reported that MDAs can also activate the transcription factor AP-1 in a c-Jun-dependent manner (27). Therefore, we tested the potential contribution of AP-1 in the MDA-mediated HIF-1 induction using MCF-7 cells stably transfected with a dominant negative c-Jun construct (dn c-Jun) that inhibits c-Jun-dependent AP-1 activity (44). As shown in Fig. 3D, cells expressing dn c-Jun induced HIF-1α in response to MDAs to a degree comparable with wild type cells, thereby discounting an involvement of c-Jun-dependent AP-1 activity in this process.

**MDA-induced HIF-1α Protein Is Transcriptionally Active**—It was of interest to determine whether the HIF-1α protein induced by MDAs was transcriptionally active. To test this, A549 cells were transfected with a HIF-1 responsive reporter plasmid that contains 3 hypoxia response elements of the iNOS gene (45). As shown in Fig. 4A, consistent with our Western results (Fig. 1), HIF-1-dependent luciferase activity was induced by treatment with MDAs. To confirm that HIF-1 reporter activity was induced in a HIF-1-specific manner, this
experiment was repeated in hepa1c1c7 cells that contain wild type Arnt, and in matched hepa1c4 cells that are unable to transactivate HIF-1-dependent genes due to a genetic defect in Arnt (46, 47). First, we determined that MDAs induce over a 2-fold induction of HIF-1 protein in hepa1c1c7 (Fig. 4B). Furthermore, this increase in HIF-1 protein correlated with over a 2-fold increase in HIF-1 reporter activity. However, in Arnt-deficient hepa1c4 cells, MDA treatment failed to increase HIF-1-dependent luciferase activity, thereby demonstrating that MDA-mediated HIF-1 activation is dependent upon and accurately represents transcriptionally active HIF-1.

We reasoned that the ability of MDAs to increase the population of transcriptionally active HIF-1 would result in the up-regulation of HIF-1 target proteins. Therefore, we examined whether the MDA-dependent increase in HIF-1 reporter activity correlated with an increase in endogenous iNOS protein, which is known to be a transcriptional target of HIF-1 (45). As shown in Fig. 4C, HIF-1α levels in hepa1c1c7 cells were induced following a 5-h treatment with the MDAs vinblastine or colchicine. However, these same agents were unable to elicit any iNOS induction in the Arnt-defective hepa1c4 cells. Therefore, the ability of MDAs to induce HIF-1 reporter activity in these cell lines reflects their ability to induce endogenous iNOS protein and is consistent with up-regulation of transcriptionally active HIF-1.

Microtubule Disruption Is Required for MDA-mediated HIF-1 Induction—While the dependence for NFκB in MDA-mediated HIF-1 induction was definitive, it remained to be determined whether microtubule disruption itself was required for HIF-1 induction. To examine this issue, MDA-mediated HIF-1 induction was assessed in both wild type Arnt and colchicine-resistant Chinese Hamster Ovary (CHO) cells. The colchicine-resistant cells contain a mutation in tubulin that alters the association of MDAs with microtubules (48, 49). As shown in Fig. 5A, while the wild type CHO cells exhibited a marked increase in HIF-1 protein following MDA treatment, the colchicine-resistant cells failed to respond to this treatment. To further verify that microtubule disruption is a component of the signaling pathway of MDAs, A549 cells were treated with l-cumicolchicine, a structurally similar, but catalytically inactive analog of colchicine. As shown in Fig. 5B, this analog failed to induce HIF-1 protein, thereby demonstrating that MDA interaction with microtubules is required for MDAs to induce HIF-1α.

MDAs Up-regulate HIF-1α Protein at the Post-transcriptional Level—Our data suggested that NFκB up-regulated HIF-1α at the transcriptional level, and we therefore examined whether HIF-1α mRNA levels were induced by MDAs. As shown in Fig. 6A, both wild type CHO cells exhibited a marked increase in HIF-1 mRNA following MDA treatment. To further verify that microtubule disruption is a component of the signaling pathway of MDAs, A549 cells were treated with γ-lumicolchicine, a structurally similar, but catalytically inactive analog of colchicine. As shown in Fig. 5B, this analog failed to induce HIF-1 protein, thereby demonstrating that MDA interaction with microtubules is required for MDAs to induce HIF-1α.
vinblastine up-regulated HIF-1α protein in the cell line containing functional VHL protein (UMRC2/VHL). However, HIF-1α was not further up-regulated in the VHL-mutated parental line containing stable HIF-1α protein. To confirm these results, transfections were performed in the well-characterized A549 cell line. As shown in Fig. 6C, vinblastine up-regulated the levels of transfected wild type HIF-1α expressed in A549 cells, while this agent failed to upregulate a mutated form of HIF-1α that is VHL-resistant. These results suggested that MDA treatment stabilized HIF-1α protein to the effects of VHL. To test this hypothesis, the stability of endogenous HIF-1α protein in A549 cells was determined either in the presence or absence of vinblastine. As shown in Fig. 6D, while endogenous HIF-1α was extremely labile in normoxic cells, with a half-life of less than 4 min, vinblastine treatment significantly stabilized the protein and extended its half-life by more than 5-fold to 20 min.

**DISCUSSION**

In this report, MDAs were used to simulate cellular responses activated by microtubule change. We demonstrate that cytoskeletal alteration mediated by a variety of microtubule-depolymerizing agents elevate protein levels of transcriptionally active HIF-1α in a pathway dependent upon NFκB activation in a variety of cell lines, suggesting that this is a basic mode of signaling universally employed by most, if not all, cell types. While MDA-mediated HIF-1 induction is not as pronounced as that elicited by hypoxia, it is significant enough to result in more than a 2-fold increase in HIF-1-regulated iNOS reporter activity and in a severalfold up-regulation of endogenous iNOS protein expression. By comparison, hypoxic stimulation of this same reporter was on the order of 3–4-fold (data not shown).

Our data conclusively demonstrate that transcription, likely mediated by NFκB activation, is a requirement for the ability of MDAs to induce HIF-1α. First, we show that transcription inhibitors interfere with the ability of MDAs to induce HIF-1α protein. Second, we demonstrate that MDAs induce NFκB activity, which correlates with the ability of these agents to induce HIF-1α levels. Third, and most compelling, treatment of cells with either a drug that inhibits NFκB or transfection with the NFκB super-repressor plasmid both abrogated the ability of MDAs to up-regulate HIF-1α protein. Finally, we demonstrate that the MDA signaling pathway for HIF-1 induction is distinct from the hypoxia-mediated pathway, in that NFκB inhibitors had no effect on reducing HIF-1α levels induced by a hypoxia mimetic, further emphasizing the unique transcriptional dependence of this mechanism.
Several reports demonstrate that drugs capable of microtubule disruption elevate NF\textsubscript{κ}B activity (24, 50–51) and in agreement with these reports, we demonstrate that MDAs induced NF\textsubscript{κ}B activity in A549 cells. However, the precise mechanism of MDA-induced NF\textsubscript{κ}B activation remains unclear. In one model, MDAs are proposed to stimulate I\textsubscript{κ}B degradation, resulting in the nuclear accumulation and subsequent activation of NF\textsubscript{κ}B (24, 52). In accordance with this model, we observed a slight increase in nuclear NF\textsubscript{κ}B upon MDA treatment (data not shown). In a second model, mechanisms potentially independent of nuclear NF\textsubscript{κ}B protein levels play a role. These mechanisms include posttranslational modification of the NF\textsubscript{κ}B protein, such as phosphorylation or acetylation of p65 (53). Interestingly, although high doses of either vinblastine or colchicine resulted in decreased NF\textsubscript{κ}B activity, no such corresponding decrease in nuclear protein levels was observed (data not shown), consistent with other reports (52). Therefore, although our data do not preclude the possibility that MDA-mediated nuclear translocation of NF\textsubscript{κ}B is an important initial event, the down-regulation of NF\textsubscript{κ}B activity without corresponding changes in nuclear p65 suggests that MDAs also initiate additional signaling events culminating in the post-translational modification of NF\textsubscript{κ}B. Although a definitive signaling pathway remains elusive, it is
notable that MDAs modulate the activities of a variety of kinases, such as protein kinases A and C, PI 3-kinase, and focal adhesion kinase (FAK), that regulate NFκB activity by phosphorylation of the p65 subunit (18, 54–55). Consistent with this hypothesis, MDAs at low, but not high concentrations, increase tyrosine phosphorylation of focal adhesion proteins such as FAK and paxillin (56). Finally, it has been demonstrated that microtubule-stabilizing agents such as paclitaxel are unable to promote activation of these same kinases (51, 57), correlating with their inability to activate NFκB and upregulate HIF-1α.

Our data demonstrate that the MDA-mediated pathway for HIF-1α induction is distinct from the hypoxia-mediated stabilization of HIF-1α. This is most clearly illustrated by data showing the dependence upon NFκB activation for the former, but not the latter pathway. However, similar to the hypoxia-mediated pathway, we find that MDAs induce HIF-1α protein at the posttranscriptional level. While we cannot absolutely rule out the possibility of increased translation of HIF-1α as an explanation of this phenomenon, in a manner similar to the effect of various growth factors (35, 58), we suggest that MDAs act by partially protecting HIF-1α protein from VHL-dependent degradation. MDAs-mediated stabilization does not render the protein completely resistant to VHL, but rather appears to engender a less efficient degradation, resulting in a 5-fold increase in half-life. Compelling evidence for this notion is provided by our finding that HIF-1α levels in a cell line lacking VHL function remained unchanged upon exposure to vinblastine. However, in a matched line with stably expressed VHL, HIF-1α accumulated in response to vinblastine, suggesting that the MDA-stabilizing effect is dependent upon VHL expression. Similarly, vinblastine elevated the level of transiently expressed wild type HIF-1α protein in A549 cells, while these agents had no effect upon a transiently expressed proline-mutated, VHL-resistant HIF-1α protein. Finally, the ability of MDAs to activate NFκB was independent of VHL status (data not shown), supporting our hypothesis that NFκB activation by MDAs occurs prior to HIF-1 accumulation. Although the complete mechanism of MDA-induced HIF-1α accumulation remains unclear, the transcriptional dependence of this pathway suggests that the mediator(s) involved may be labile. Evidence for a labile mediator is further provided by our data (Fig. 3C) demonstrating that transcriptional inhibition of MDA-stabilized HIF-1α results in a moderate decrease in protein levels within the first hour. This labile mediator(s) may modify HIF-1α, or another protein involved VHL-HIF association, so as to render HIF-1α less susceptible to VHL-mediated degradation.

Tumor cell invasion and metastasis, hallmarks of the tumorigenic process, involve microtubule reorganization. We demonstrate that MDA-mediated activation of NFκB and subsequent induction of HIF-1α is initiated by and depends upon microtubule depolymerization. While the specific role NFκB may play in invasion and metastasis is unclear, several reports document overexpression and/or hyperactivity of NFκB in cancer lines (59) and tissues (60). Inhibition of NFκB correlates with suppression of metastasis and invasion (61, 62), down-regulation of VEGF mRNA (63), and suppression of angiogenesis (62), effects, which may be mediated through regulation of HIF-1α. Given that HIF-1α is overexpressed in a majority of tumors (12), the data in this study suggest that HIF-1α is among the pro-oncogenic factors induced by NFκB.

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