Evidence for a Role of p38 Kinase in Hypoxia-inducible Factor 1-independent Induction of Vascular Endothelial Growth Factor Expression by Sodium Arsenite*

Monique C. A. Duyndam‡, Saskia T. M. Hulscher, Elsken van der Wall, Herbert M. Pinedo, and Epie Boven

From the Department of Medical Oncology, Vrije Universiteit Medical Center, Amsterdam 1081 HV, The Netherlands

Recently we have demonstrated that sodium arsenite induces the expression of hypoxia-inducible factor 1α (HIF-1α) protein and vascular endothelial growth factor (VEGF) in OVCAR-3 human ovarian cancer cells. We now show that arsenic trioxide, an experimental antitumor agent, exerts the same effects. The involvement of phosphatidylinositol 3-kinase 3-kinase and mitogen-activated protein kinase (MAPK) pathways in the effects of sodium arsenite was investigated. By using kinase inhibitors in OVCAR-3 cells, both effects of sodium arsenite were found to be independent of phosphatidylinositol 3-kinase and p44/p42 MAPKs but were attenuated by inhibition of p38 MAPK. A role for p38 in the regulation of HIF-1α and VEGF expression was supported further by analysis of activation kinetics. Experiments in mouse fibroblast cell lines, lacking expression of c-Jun N-terminal kinases 1 and 2, suggested that these kinases are not required for induction of HIF-1α protein and VEGF mRNA. Unexpectedly, sodium arsenite did not activate a HIF-1-dependent reporter gene in OVCAR-3 cells, indicating that functional HIF-1 was not induced. In agreement with this hypothesis, up-regulation of VEGF mRNA was not reduced in HIF-1α<sup>−/−</sup> mouse fibroblast cell lines. Altogether, these data suggest that not HIF-1, but rather p38, mediates induction of VEGF mRNA expression by sodium arsenite.

Elevated expression of VEGF in tumor cells can be the result of environmental factors, such as hypoxia, or mutations in oncogenes or tumor suppressor genes that regulate growth factor signal transduction pathways (3–9). Many stimuli, including hypoxia, growth factors, hormones, and oxidative stressors, can increase VEGF expression in tumor cells in vitro (6, 10–14). In all mentioned cases, increased VEGF expression can be caused in part by increased VEGF gene transcription mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1) (4–7, 11, 13–15). HIF-1 binds to a hypoxia-responsive element (HRE) located within the VEGF promoter (11).

HIF-1 is composed of two subunits, HIF-1α and HIF-1β. The activity of HIF-1 is regulated mainly by the expression and activity of the HIF-1α subunit. Although HIF-1β protein is rather stable and readily detected in the nucleus of most normoxic cells, HIF-1α protein is often hardly detectable because of rapid degradation by the ubiquitin–proteosome system (16–18). Hypoxia increases the level of HIF-1α protein by inhibiting its ubiquitination and degradation (19). Accumulation of HIF-1α protein can also be observed in stimulated normoxic cells (5, 8, 9, 13–15). HIF-1α is subsequently translocated to the nucleus, where it can dimerize with HIF-1β to form the HIF-1 complex (20). To be fully active, HIF-1α requires interaction(s) with coactivators or/and transcription factor(s) (20–23). The activated protein-1 transcription factor family member c-Jun interacts with HIF-1α and is suggested to cooperate with HIF-1 in the induction of VEGF expression by hypoxia (23).

The stabilization and transcriptional activation of the HIF-1α protein involve changes in its phosphorylation state. Activation of the lipid kinase phosphatidylinositol 3-kinase (PI3K), and/or its downstream target the protein-serine/threonine kinase Akt, can result in the phosphorylation and stabilization of HIF-1α under hypoxic and normoxic conditions (8, 24). In addition, inhibition of PI3K activity has been shown to reduce the transactivation function of HIF-1α in hypoxic cells (25). So far, there is no evidence that PI3K and Akt can phosphorylate HIF-1α directly. Other pathways that regulate HIF-1α phosphorylation involve members of the mitogen-activated protein kinase (MAPK) family (26). In hypoxic and in stimulated normoxic cells, p44/p42 MAPK (extracellular signal-regulated kinase [ERK]-1 and ERK-2) and p38 MAPK enhance the transactivation function of HIF-1α (24, 25, 27). This may occur through direct phosphorylation because these kinases have been shown to phosphorylate HIF-1α in vitro (28, 29). In some cases, p44/p42 MAPK and p38 can also influence HIF-1α protein induction (8, 30). The stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs) do not phosphorylate HIF-1α in vitro, but may indirectly regulate HIF-1α-mediated transcription of VEGF under hypoxia through phosphorylating the transcription factor c-Jun (18, 23).
The stabilization and transcriptional activation of HIF-1α may also involve alterations in its redox state. Evidence is provide that changes in the levels of reactive oxygen species (ROS) may play a role in HIF-1α protein induction and HIF-1α transactivation (14, 30–32). Increased levels of ROS are suggested to mediate PI3K activation under hypoxia as well as under normoxia (32, 33). The levels of ROS may also directly or indirectly influence the redox status of cysteine residues in the transactivation domains of HIF-1α, which can affect interactions with transcriptional coactivators (21, 22).

In a previous study on the role of oxidative stress in the regulation of VEGF, we showed that sodium arsenite (NaAsO₂) induces HIF-1α protein and VEGF mRNA and protein levels in the human ovarian cancer cell lines OVCAR-3 and H134 (34). Arsenite induces oxidative stress by binding to thiol groups of cellular proteins and by increasing the production of ROS. Because the effects of sodium arsenite on HIF-1α protein and VEGF expression are independent of increased ROS production, we hypothesized that they may be mediated through binding of arsenite to thiol (SH) groups of the HIF-1α protein itself or of components of signal transduction pathways involved in HIF-1 or VEGF regulation (34).

Our findings with sodium arsenite are of clinical relevance. Several cytotoxic agents in cancer treatment are susceptible of interacting with thiol groups of cellular proteins. Moreover, arsenic trioxide (As₂O₃), another trivalent arsenic compound, has potential as an anticancer agent (35). At low dosages (1–10 μM), arsenic trioxide has a significant cytotoxic effect on human ovarian cancer cell lines and is suggested to be a useful agent for the treatment of ovarian cancer (36). Therefore, we now compared the potency of arsenic trioxide with that of sodium arsenite to induce HIF-1α protein and VEGF mRNA and protein levels in the OVCAR-3 human ovarian cancer cell line. We also investigated the role of the PI3K/Akt pathway and of MAPK family members in sodium arsenite-induced HIF-1α protein accumulation and VEGF expression. Furthermore, we examined whether up-regulation of VEGF mRNA expression by sodium arsenite was mediated by HIF-1.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Sodium arsenite, arsenic trioxide, wortmannin, glutathione (GSH), N-acetylcysteinine (NAC), and buthionine-sulfoximine (BSO) were purchased from Sigma. PD98059, SB203580 and SB202190 were purchased from Calbiochem.

**Cell Culture and Cell Treatments**—OVCAR-3 human ovarian cancer cells and Jnk1/2−/−Jnk2−/−, Jnk1−/−Jnk2−/−, and HIF-1α−/− mouse fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. The stabilization and transcriptional activation of HIF-1α may also involve alterations in its redox state. Evidence is provide that changes in the levels of reactive oxygen species (ROS) may play a role in HIF-1α protein induction and HIF-1α transactivation (14, 30–32). Increased levels of ROS are suggested to mediate PI3K activation under hypoxia and by normoxia (32, 33). The levels of ROS may also directly or indirectly influence the redox status of cysteine residues in the transactivation domains of HIF-1α, which can affect interactions with transcriptional coactivators (21, 22).

**Preparation of Cell Extracts for Western Blot Analysis**—Cells were washed once with ice-cold phosphate-buffered saline and lysed by scraping with a rubber policeman in 250 μl of radioimmunoassay precipitation assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 1% sodium deoxycholate) for Western blots in 450 μl of Elisa buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40) for ELISA. Both lysis buffers were supplemented with 50 mM NaF, 1 mM Na₃VO₄, 1.0 mM phenylmethylsulfonyl fluoride, 0.5 mM trypsin inhibitor, and 0.5 μg/ml leupeptin. After a 15-min incubation period on ice, the extracts were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C and stored at −70 °C. Isolation of nuclear and cytoplasmic protein fractions from OVCAR-3 cells in the subcellular fractionation experiment was performed as described previously (40). Protein concentrations were determined by the Coomassie Plus Protein assay (Pierce).

**Western Blotting**—Equal amounts of protein cell extracts were resolved in SDS-polyacrylamide gels and transferred electrophoretically onto polyvinylidene difluoride membrane (Immobilon). Membranes were blocked for 1 h in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.025% Tween 20) and 5% milk and incubated overnight with anti-VEGF antibodies: rabbit polyclonal antiserum against phospho-Akt-1 (Ser473), phospho-p44/p42 MAPK (Thr183/Tyr185), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-c-Jun (Ser63), p44/p42 MAPK, SAPK/JNK, p38 MAPK, and a horseradish peroxidase-coupled anti-rabbit antiserum was purchased from New England Biolabs. Mouse monoclonal antisera to HIF-1α was purchased from Novus Biologicals/AbCam (Cambridge, U. K.) and BD Transduction Laboratories (Alphen a/d Rijn, The Netherlands). The sheep polyclonal antiserum directed against Akt-1 was from Upstate Biotechnology Inc. Rabbit polyclonal antisera directed against c-Jun (H-79) and Raf-1 (C12) were purchased from Santa Cruz Biotechnology. The mouse monoclonal antisera against human p53 (DO-7) and the horseradish peroxidase-coupled anti-mouse serum were purchased from DAKO (Glostrup, Denmark). The horseradish peroxidase-coupled anti-rabbit serum was purchased from Calbiochem.

**RNase Protection Assay**—Generation of human γ-actin and VEGF...
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VEGF164 and digestion mixtures were subjected to gel electrophoresis, and the mean relative luciferase activity was assessed as described by Sambrook et al. (47). Luciferase activity was determined by mixing 30 μl of cell extract with 100 μl of luciferase assay reagent (Promega) and subsequent measurement of luminescence. The RNase protection assay was carried out as described (34). Hybridization of total RNA to the 301-nucleotide human VEGF165 antisense probe and the 228-nucleotide murine VEGF164 antisense probes has been described elsewhere (34). pcDNA3 vectors were generated as follows. Total RNA from Jnk1−/− and Jnk2−/− mouse fibroblasts was reversed transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Murine VEGF164 cDNA (nucleotides 454–648) was amplified with the forward primer 5'-ATCAACACGTTAGCAGAGTAGCTGGATGC-3' and the reversed primer 5'-GCAAACCTCGGACCTGCTACATGC-3'. The RNase protection assay was carried out as described (34). Hybridization of total RNA to the 185-nucleotide murine VEGF164 antisense probe and the 228-nucleotide murine VEGF164 antisense probe was carried out as described (34).

**Results**

**High Concentrations of Arsenic Compounds Induce HIF-1α Protein and VEGF Expression in OVCAR-3 Cells**—We compared the effects of different concentrations of arsenic trioxide with those of sodium arsenite on HIF-1α protein, VEGF mRNA, and VEGF protein expression in OVCAR-3 cells (Fig. 1). As can be seen in Fig. 1A, high concentrations of arsenic trioxide (100 and 50 μM) could indeed induce HIF-1α protein after 4 and 8 h in OVCAR-3 cells, but to a much lesser extent than an equimolar concentration of sodium arsenite (100 μM). We also tested the effect of 8- and 24-h exposure periods to 10 μM arsenic trioxide. This treatment did not induce detectable changes in HIF-1α protein expression in OVCAR-3 cells.

The effects of arsenic trioxide on VEGF mRNA expression were assessed by the RNase protection assay (Fig. 1B). As determined by measurement of the signal intensities of the 252-nucleotide VEGF165- and 130-nucleotide VEGF antisense fragments, VEGF165 mRNA levels in OVCAR-3 cells were increased 3- and 5-fold after 4 and 8 h of exposure to 100 μM arsenic trioxide, respectively. A 2- and 3-fold increase in VEGF165 mRNA expression was observed after 4 and 8 h of exposure to 50 μM arsenic trioxide. An 8-h exposure period to low concentrations of arsenic trioxide (10 μM) did not influence the level of VEGF165 mRNA, whereas a weak elevation (1.5-fold) was observed after 24 h of exposure. In agreement with the effects on HIF-1α protein, arsenic trioxide at 100 μM was a less potent inducer of VEGF165 mRNA than sodium arsenite. 100 μM sodium arsenite increased the level of VEGF165 mRNA up to 6- and 7-fold after 4 and 8 h of exposure.

We next assessed whether induction of VEGF mRNA levels by arsenic trioxide resulted in an increased production of VEGF protein. OVCAR-3 cells were grown overnight, and arsenic trioxide and sodium arsenite were added to the conditioned medium. VEGF concentrations in the conditioned medium or in cell lysates of treated and nontreated cells were measured by ELISA. As can be seen in Fig. 1C, VEGF protein levels in the conditioned medium were increased significantly after 8 h of exposure to 100 and 50 μM of arsenic trioxide compared with the levels in the medium of control cells (p < 0.05). In the lysates, increased production of VEGF was evident after 4 h of incubation and was more pronounced after 8 h (p < 0.05). As expected, a higher increase in VEGF protein concentrations in conditioned medium and lysate was observed after treatment with 100 μM sodium arsenite. Note that a weak elevation of VEGF protein levels was detected in conditioned medium and lysate of cells that were exposed to 10 μM arsenic trioxide for 24 h. These results clearly show that arsenic trioxide induces HIF-1α protein accumulation and VEGF expression in OVCAR-3 cells, albeit less potently than sodium arsenite. In addition, these results indicate that long exposure to a low, clinically relevant concentration of arsenic trioxide can weakly increase VEGF165 mRNA and protein expression in the absence of a detectable change in the level of HIF-1α protein expression.

**Induction of HIF-1α Protein and VEGF Expression by Sodium Arsenite Is Not Mediated through PI3K**—Because it has been suggested that sodium arsenite can activate PI3K in some cell types, we investigated the role of this pathway in sodium arsenite-induced HIF-1α protein accumulation and VEGF expression in OVCAR-3 cells (48). PI3K-mediated activation of Akt-1, the most frequently studied isoform of Akt, involves phosphorylation on its amino acid residues Ser473 and Thr308. We first monitored the phosphorylation of Akt-1 upon sodium arsenite treatment in OVCAR-3 cells by Western blotting with an antiserum recognizing Ser473-phosphorylated Akt-1. As can be seen in Fig. 2A, OVCAR-3 cells showed a relatively high basal level of phospho-Akt-1. After 15 min of exposure to 100 μM sodium arsenite, Akt-1 phosphorylation on Ser473 was increased, which was sustained until at least 4 h of exposure. After 8 h, the levels of phospho-Akt-1 were decreased to basal. The same results were obtained with an antiserum directed against Thr308 (data not shown). The levels of total Akt-1 were
unchanged upon sodium arsenite treatment for up to 8 h. We examined further the involvement of PI3K in sodium arsenite-induced Akt-1 phosphorylation by assessing the effects of the PI3K inhibitor wortmannin. Fig. 2B shows that pretreatment with 100 nM wortmannin completely blocked induction of Akt-1 phosphorylation by sodium arsenite. Wortmannin alone did not significantly affect the basal level of phospho-Akt-1. Again, the total levels of Akt-1 protein remained constant. These results suggest that sodium arsenite indeed activates the PI3K/Akt-1 pathway in OVCAR-3 cells.

We next investigated the effect of wortmannin on sodium arsenite-induced HIF-1α protein accumulation by analyzing HIF-1α protein levels in the extracts of Fig. 2A by Western blot. In sharp contrast to the phosphorylation of Akt-1, induction of HIF-1α protein by sodium arsenite was completely unaffected by 100 nM wortmannin (Fig. 2C). Pretreatment with wortmannin concentrations as high as 500 nM only weakly inhibited HIF-1α protein accumulation. Analysis of total RNA by the RNase protection assay in parallel samples revealed that neither sodium arsenite-induced VEGF165 mRNA levels nor the basal VEGF165 mRNA levels in OVCAR-3 cells were influenced by pretreatment with 100 and 500 nM concentrations of wortmannin (Fig. 2D). These results strongly suggest that induction of HIF-1α protein and VEGF expression by sodium arsenite is independent of the PI3K/Akt-1 pathway in OVCAR-3 cells.

Sodium Arsenite activates p44/p42 MAPK, SAPK/JNK, and p38 with Different Kinetics in OVCAR-3 Cells—Depending on the cell type, sodium arsenite can activate different MAPK family members (49), which may contribute to HIF-1α protein accumulation and VEGF expression. Therefore, we first monitored the activity of p44/p42 MAPK, SAPK/JNK, and p38 upon sodium arsenite treatment in OVCAR-3 cells by detection of their phosphorylated, activated forms on Western blots.

As can be seen in Fig. 3, nonstimulated OVCAR-3 cells showed strong phosphorylation of p44/p42 MAPK with different kinetics. Pretreatment with sodium arsenite for 5 min increased the phosphorylation of p44/p42 MAPK, SAPK/JNK, and p38 in OVCAR-3 cells. These results suggest that sodium arsenite activates different MAPK family members in OVCAR-3 cells.
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FIG. 2. Effect of wortmannin on sodium arsenite-induced Akt-1 phosphorylation, HIF-1α protein accumulation, and VEGF expression in OVCAR-3 cells. OVCAR-3 cells were exposed to 100 μM sodium arsenite in the absence or presence of 100 or 500 nM wortmannin or to 100 or 500 nM wortmannin alone. Wortmannin was added 1 h before the addition of sodium arsenite. After the indicated periods of exposure to sodium arsenite, cells were lysed, and protein and total RNA were extracted. A and B, the phosphorylation of Akt-1 was monitored by subjecting 100 μg of protein to SDS-gel electrophoresis (8% gel) followed by Western blotting with an antisera specific to Ser(Tyr)473. The blots were stripped and reprobed with a total Akt-1 antisera. C, HIF-1α protein levels were examined by subjecting 100 μg of protein to SDS-gel electrophoresis (7.5% gel) followed by Western blotting with a HIF-1α-directed antisera. D, VEGF165 mRNA levels were analyzed by RNase protection as in B.

FIG. 3. Effect of sodium arsenite on the phosphorylation of MAPK family members in OVCAR-3 cells. OVCAR-3 cells were exposed to 100 μM sodium arsenite for the indicated time periods. 25 μg of protein was subjected to SDS-gel electrophoresis (10% gel) and Western blotting. Phosphorylation of p44/p42 MAPK, JNK1/2 (p46, p54), and p38 was detected using phospho-antiseras. The blots were stripped and reprobed with antisera recognizing both nonphosphorylated and phosphorylated forms of p44/p42 MAPK, JNK1/2 (p46, p54), and p38.

detectable until at least 1 h of exposure. The phosphorylation of both kinases returned to basal levels after 2 h and was even reduced to lower levels after 4 and 8 h. Sodium arsenite also induced the activity of JNK1, JNK2, and p38, albeit with different kinetics. A strong increase in the phosphorylation of JNK1, JNK2, and p38 was observed after 1 h of sodium arsenite treatment, and increased phosphorylation of all three kinases was prolonged until at least 8 h of treatment. Changes in phosphorylation of p42/p44 MAPK, JNK1, JNK2, and p38 were not accompanied by alterations in the levels of these proteins. These data indicate that sodium arsenite activates p44/p42 MAPK, JNK1, JNK2, as well as p38 with different kinetics in OVCAR-3 cells. Activation of p44/p42 MAPK occurred rapidly but was only moderate and transient. In contrast, activation of JNK1, JNK2, and p38 was slower but more potent and sustained.

Sodium Arsenite-induced HIF-1α Protein Accumulation and VEGF Expression Are Attenuated by Inhibitors of p38 MAPK Activity—To further examine the involvement of p44/p42 MAPK and p38 in the induction of HIF-1α protein and VEGF expression by sodium arsenite, we analyzed the effect of the kinase inhibitors PD98059, SB202190, and SB203580 in OVCAR-3 cells. PD98059 is a specific inhibitor of MAPK/ERK kinase (MEK)-1 and MEK-2, which phosphorylate and activate p44/p42 MAPK. SB202190 and SB203580 directly inhibit the activity of p38 by binding to its ATP binding domain.

FIG. 4, A–C, shows that induction of HIF-1α protein and VEGF165 mRNA expression after 4 and 8 h of exposure to 100 μM sodium arsenite was slightly, but not significantly, reduced by pretreatment with high concentrations of PD98059 (50 μM). The basal level of HIF-1α protein and VEGF165 mRNA was also hardly affected by this agent. PD98058 was functional in inhibiting p44/p42 MAPK activity at the concentrations used, as demonstrated by the reduced level of phospho-p44/p42 MAPK in the presence and absence of sodium arsenite (Fig. 4D). In fact, phospho-p44/p42 MAPK levels were even below basal and hardly detectable after 4 and 8 h of sodium arsenite and PD98059. These data indicate that p44/p42 MAPK does not regulate induction of HIF-1α protein and VEGF expression in OVCAR-3 cells.

Pretreatment with the p38 inhibitors SB202190 and SB203580 could potently inhibit induction of HIF-1α protein and VEGF mRNA expression by sodium arsenite (Fig. 4, A–C). As assessed at 4 and 8 h after the addition of 100 μM sodium arsenite, both effects were almost completely attenuated by a 20 μM concentration of each inhibitor. Significant inhibition of HIF-1α protein accumulation and VEGF mRNA expression was also observed by pretreatment with 5 μM SB202190 (data not shown) and SB203580 (Fig. 4, A–C). Treatment with the two inhibitors alone did not significantly influence the basal level of HIF-1α protein and VEGF165 mRNA in OVCAR-3.

In some cell types, high concentrations (20–100 μM) of SB202190 or SB203580 can inhibit the activity of JNKs (51). Activated JNKs are known to enhance the activity of the transcription factor c-Jun through the phosphorylation of two serine residues at positions 63 and 73 (26). To examine whether JNK activity was influenced by SB203580 in sodium arsenite-treated OVCAR-3 cells, we analyzed the phosphorylation of c-Jun on Ser73 and the total level of c-Jun by Western blotting. Fig. 4E shows that phosphorylated c-Jun proteins were not detectable in nonstimulated OVCAR-3 cells. After 2 h of sodium arsenite treatment, phosphorylation of c-Jun protein was clearly observed and was increased further until at least 8 h of exposure. Consistent with findings that phosphorylation on Ser73 and Ser63 decreases the electrophoretic mobility of the c-Jun protein (52), slower migrating forms of c-Jun were detectable after sodium arsenite treatment. The total level of c-Jun protein was also increased by sodium arsenite. Pretreatment with 20 μM SB203580 did not prevent the phosphorylation of c-Jun on Ser73 in OVCAR-3 cells upon exposure to sodium arsenite (Fig. 4E). Hyperphosphorylated, retarded c-Jun protein band(s) were clearly detectable. The observed de-
sequent normalization of VEGF165 values against sodium arsenite, cells were lysed, and protein and RNA were extracted.

The kinase inhibitors were added 1 h before the addition of sodium arsenite, which reduces intracellular GSH—We have shown previously that the level of intracellular GSH is critical for induction of HIF-1α protein and VEGF expression by sodium arsenite in OVCAR-3 cells (34). Pretreatment with agents that elevate intracellular GSH levels, such as GSH and NAc (a precursor of GSH), attenuate sodium arsenite-induced HIF-1α accumulation and VEGF expression, whereas depletion of intracellular GSH by pretreatment with BSO potentiates both effects (34). The latter agent causes GSH depletion by inhibiting γ-glutamylcysteine synthetase, an enzyme involved in the synthesis of GSH (53).

In light of our findings with the p38 inhibitors that p38 may be an upstream regulator of HIF-1α protein and VEGF expression, we assessed whether activation of p38 by sodium arsenite was also modulated by exposure to GSH, NAc, and BSO prior to the addition of sodium arsenite in OVCAR-3 cells. As a control, the level of the HIF-1α protein was analyzed in parallel in the same extracts. Fig. 5A shows that pretreatment with 10 and 20 mM GSH and NAc almost completely attenuated sodium arsenite-induced phosphorylation of p38 in OVCAR-3 cells. Differences in the signal of the phospho-p38 band were not caused by changes in the total level of p38 because these remained almost constant under all circumstances. The basal level of p38 phosphorylation appeared not to be influenced by pretreatment with 20 mM concentrations of the antioxidants alone. In agreement with our previous findings (34), GSH and NAc pretreatment also attenuated HIF-1α protein induction, whereas a minor effect was observed on the basal level of HIF-1α protein.

To examine the effect of GSH depletion on p38 phosphorylation, OVCAR-3 cells were pretreated with 500 μM BSO for 16 h before the addition of a lower, suboptimal concentration of sodium arsenite (30 μM). Because sodium arsenite reduces intracellular GSH levels by itself, the effect of BSO is more pronounced when OVCAR-3 cells are exposed to a suboptimal concentration of this agent. Fig. 5B shows that pretreatment with BSO clearly potentiated the phosphorylation of p38 after 4, 6, and 8 h of exposure to sodium arsenite, without affecting the total levels of this protein. Pretreatment with 500 μM BSO alone for a period of 24 h hardly affected the basal levels of p38 phosphorylation. It should be mentioned that potentiation of p38 activity in the presence of BSO was already observed after 4 h, whereas potentiation of HIF-1α protein accumulation was not detected until 6 h of exposure to sodium arsenite. Thus, the p38 response in GSH-depleted OVCAR-3 cells upon sodium arsenite treatment precedes the response of HIF-1α. This re-
The lack of JNK activity in the two cell line in which one of the four mRNA levels in two mouse fibroblast cell lines that were deficient of Jnk1 and Jnk2 are not essential for induction of HIF-1α protein and/or VEGF164 mRNA expression by sodium arsenite. In two leftmost lanes, the full-length Jnk2-actin mRNAs. The levels of VEGF164 mRNA in the two Jnk1+/− Jnk2−/− cell lines were increased 3–4-fold after 4 h of treatment with 100 μM sodium arsenite. In Jnk1+/− Jnk2−/− fibroblasts, induction of VEGF mRNA expression by sodium arsenite was also detectable, but was less pronounced (2-fold). Elevated levels of VEGF164 mRNA were still observed after 8 h of exposure to arsenite in all three cell lines. In summary, our results in mouse fibroblast cell lines demonstrate that induction of HIF-1α protein and VEGF mRNA levels by sodium arsenite is not restricted to...
human ovarian cancer cells. More importantly, the data suggest that JNK1 and JNK2 are not essential for these effects.

**HIF-1α Protein Is Translocated to the Nucleus in Sodium Arsenite-treated OVCAR-3 Cells**—So far, it is unclear whether induction of HIF-1α protein by sodium arsenite leads to the formation of functional HIF-1. Because the translocation of HIF-1α to the nucleus is an important regulatory event in the activation of HIF-1, we first examined the subcellular localization of HIF-1α in nontreated and sodium arsenite-treated OVCAR-3 cells. Because hypoxia has been shown to induce efficient nuclear translocation of HIF-1α in several cell types (20), we assessed the subcellular distribution of HIF-1α in hypoxic OVCAR-3 cells as a control. Cytoplasmic and nuclear protein fractions were extracted from nontreated OVCAR-3 cells and from cells exposed to 100 μM sodium arsenite or hypoxia (1% O₂) for 6 h, and identical amounts of protein extract were subjected to SDS-PAGE and Western blotting in addition, whole cell extracts were prepared and analyzed in parallel. Fig. 7 shows that a low level of HIF-1α protein expression was detected in whole cell extracts of nontreated OVCAR-3 cells and that the level of HIF-1α protein was potently induced upon exposure to sodium arsenite as well as to hypoxia (34). A low level of HIF-1α protein was observed in the nuclear fraction but not in the cytoplasmic fraction of nontreated OVCAR-3 cells. Much higher levels of HIF-1α were observed in the nuclear fractions as well as in the cytoplasmic fractions of OVCAR-3 cells that were exposed to sodium arsenite and hypoxia. As a control for the fractionation procedure, we also analyzed the levels of proteins with a known subcellular localization (Fig. 7). As assessed in whole cell extracts, the levels of the cytoplasmic proteins Raf-1 and β-actin the nuclear proteins topoisomerase I and p53 (mutant conformation) were unaffected by treatment with sodium arsenite and hypoxia. Raf-1 and β-actin were indeed present primarily in the cytoplasmic fractions, whereas topoisomerase I and p53 were detected mainly in the nuclear fractions. These control tests confirmed that the fractionation procedure was valid. Altogether, these data strongly indicate that HIF-1α is transported to the nucleus in nontreated OVCAR-3 cells and that HIF-1α protein accumulates in the nucleus and in the cytoplasm of OVCAR-3 cells upon exposure to sodium arsenite and hypoxia.

**Induction of HIF-1α Protein by Sodium Arsenite May Not Lead to the Formation of Functional HIF-1 in OVCAR-3 Cells**—To examine further the functional activity of HIF-1α upon sodium arsenite treatment, OVCAR-3 cells were transiently transfected with a 5xHREpGL3 luciferase reporter gene construct containing five HRE binding sites from the human VEGF promoter in front of the SV40 minimal promoter. The empty pGL3 promoter construct, containing only the SV40 minimal promoter in front of the luciferase gene, was transfected as a negative control. We also transfected a 5xjun2pGL3 reporter construct, which contains five copies of the jun2 element from the c-jun promoter in front of the SV40 minimal promoter. The jun2 element binds c-Jun/ATF-2 heterodimers and is suggested to mediate induction of c-jun gene expression by agents that induce different types of cellular stress, including sodium arsenide (54, 55). As mentioned earlier, c-Jun is a substrate of JNKs, whereas ATF-2 is phosphorylated and activated by JNKs as well as by p38 (26). Because c-jun mRNA levels were elevated in sodium arsenite-treated OVCAR-3 cells (data not shown), we transfected the 5xjun2pGL3 reporter construct as a positive control.

As can be seen in Fig. 8, the 5xHREpGL3 construct was not induced by 50 and 100 μM sodium arsenite as measured after 6 h of exposure. To exclude the possibility that the 5xHREpGL3 construct was not functional, transfected OVCAR-3 cells were exposed for the same period of 6 h to hypoxia in parallel. Note that HIF-1α protein expression was strongly induced after 6 h of exposure to hypoxia as well as after 6 h of exposure to 100 μM sodium arsenite (Fig. 7). In agreement with findings in other cell types, the expression of the 5xHREpGL3 reporter gene was significantly greater (7-fold) than that of the parental pGL3 construct in hypoxic OVCAR-3 cells. This suggests that the 5xHREpGL3 construct is functional in mediating transcriptional responses through HIF-1. The 5xjun2pGL3 construct was induced ~2-fold after treatment with 50 and 100 μM sodium arsenite. This activation is actually 3-fold when normalized for the activity of the parental pGL3 construct under the same conditions. Although the kinetics of activation of c-Jun/ATF-2 and HIF-1 upon sodium arsenite treatment may differ, our findings with the 5xjun2pGL3 construct suggest that the assay conditions used allow detection of increased expression of pGL3 reporter constructs in arsenite-treated OVCAR-3. Altogether, these data indicate that induction of HIF-1α protein by sodium arsenite may not lead to the formation of functional HIF-1 in OVCAR-3 cells.
HIF-1α Is Not Essential for Induction of VEGF Expression by Sodium Arsenite in Mouse Fibroblasts—To examine further the role of HIF-1 in sodium arsenite-induced VEGF mRNA expression, we have studied the effect of this agent on VEGF mRNA expression in a wild type and a HIF-1α-deficient mouse fibroblast cell line (39, 56). As shown in the RNase protection assay in Fig. 9, unstimulated HIF-1α−/− as well as HIF-1α−/− fibroblasts express a very low level of VEGF164 mRNA. After 4 h of exposure to sodium arsenite, VEGF164 mRNA levels were elevated 2.5-fold in HIF-1α−/− cells. In HIF-1α−/− cells, the increase in VEGF164 mRNA at this time point was even higher, 3.5-fold. In both cell types, the β-actin mRNA levels were not altered upon sodium arsenite treatment. These findings in mouse fibroblasts strongly suggest that HIF-1α(α) does not play a role in the up-regulation of VEGF mRNA expression by sodium arsenite and support our suggestive data in OVCAR-3 cells that induction of HIF-1α protein by sodium arsenite does not result in the generation of functional HIF-1.

**DISCUSSION**

We have demonstrated previously that sodium arsenite induces HIF-1α protein as well as VEGF mRNA and protein expression in the human ovarian cancer cell lines OVCAR-3 and H134 (34). Here, we show that 50 and 100 μM arsenic trioxide, an experimental anticancer agent, exerts the same effects in OVCAR-3 cells. Although treatment of OVCAR-3 cells with 10 μM arsenic trioxide for a relatively long period of exposure (24 h) did not influence HIF-1α protein levels, a weak elevation was observed in the expression of VEGF165 mRNA (1.5-fold). The levels of VEGF protein in conditioned medium and in cell lysate were increased 3- and 2-fold, respectively. These findings suggest that at low, clinically achievable concentrations of arsenic trioxide, ovarian cancer cells may respond with up-regulation of VEGF protein.

The finding that arsenic trioxide induces HIF-1α protein and VEGF expression was not unexpected because different arsenic compounds containing trivalent arsenic have been shown to exert similar biological effects (49). At least part of these effect are believed to be caused by the ability of trivalent arsenic to bind to SH groups of important cellular (signaling) proteins. Both sodium arsenite and arsenic trioxide can activate PI3K and/or the MAPK family members p44/p42 MAPK, SAPKs/JNKS, and p38 (48, 49). We demonstrated that these kinases were indeed activated by sodium arsenite in OVCAR-3 cells. Our experiments with the PI3K inhibitor wortmannin and the p44/p42 MAPK inhibitor PD98059 strongly suggest that sodium arsenite-induced HIF-1α protein and VEGF mRNA expression are independent of PI3K/Akt-1 and p44/p42 MAPK. Interestingly, phenylarsine oxide, another trivalent arsenic compound, can also induce HIF-1α protein through a PI3K/Akt-1-independent mechanism (57).

Recently, it was suggested that the transcription factor c-Jun cooperates with HIF-1 in the activation of VEGF and that JNKs mediate this activation by phosphorylating c-Jun on Ser63 and Ser73 (23). We showed induction of HIF-1α protein and VEGF165 mRNA in immortalized Jnk1−/− Jnk2−/− and Jnk1−/− Jnk2−/− mouse fibroblast cell lines by sodium arsenite, which did not involve c-Jun phosphorylation on Ser63 and Ser73. Thus, our data provide strong evidence that JNK activity is not essential for the effects of sodium arsenite on HIF-1α and VEGF.

The p38 MAPK inhibitors SB202190 and SB203580 completely abolished induction of HIF-1α protein and VEGF165 mRNA expression by sodium arsenite in OVCAR-3 cells. A role of p38 as an upstream activator in the effects of sodium arsenite on HIF-1α and VEGF was supported by additional observations. First, activation of p38 was observed after 1 h of exposure to sodium arsenite and clearly preceded the induction of HIF-1α protein and VEGF165 mRNA levels (observed after 4 and 2 h, respectively) (34). Second, we found p38 kinase activation as well as HIF-1α protein and VEGF165 mRNA expression to be co-modulated by the level of intracellular GSH (34). Elevation of intracellular GSH by pretreatment with GSH or NAc (a precursor for GSH) inhibited the effects of sodium arsenite on HIF-1α protein and VEGF165 mRNA expression in OVCAR-3 cells, whereas depletion of intracellular GSH by BSO showed the reverse (34). We demonstrated here that pretreatment with GSH, NAc, and BSO influenced the effects of sodium arsenite on p38 activity in the same manner. Thus, p38 may act as a mediator in the modulation of HIF-1α accumulation and VEGF expression by intracellular GSH. Third, we have transient cotransfected OVCAR-3 cells with an expression vectors containing either a puromycin resistance gene or a gene encoding an unphosphorylatable dominant-negative mutant of p38 (DNp38). As assessed by real time quantitative PCR, induction of VEGF165 mRNA by sodium arsenite after 3 days of selection with puromycin was partly inhibited (20–40%) upon cotransfection of DNp38 in three independent experiments. The partial inhibition is likely the result of suboptimal selection of transfected cells with puromycin. Upon stable cotransfection, 19 puromycin-resistant OVCAR-3 clones did not show detectable expression of DNp38, indicating that overexpression of DNp38 may inhibit OVCAR-3 cell growth. For further studies, the generation of OVCAR-3 cells with inducible expression of DNp38 may be required.

Although a role for p38 in the regulation of HIF-1α protein levels under normoxia has been suggested in some studies, this kinase has mainly been implicated in the regulation of HIF-1α transactivation (25, 29). The mechanism by which p38 exerts its effect on HIF-1α, however, is still unclear. We have provided evidence that sodium arsenite induces the level of HIF-1α protein by inhibiting its degradation (34). The degradation of HIF-1α is controlled by a small domain of 200 amino acids (amino acids 401–603), called the oxygen-dependent degradation domain (17). Interestingly, this oxygen-dependent degradation domain contains three serine residues (at positions 581, 589, and 594) which may be putative targets for direct phosphorylation by p38. The oxygen-dependent degradation domain mediates the interaction of HIF-1α with the tumor suppressor...
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protein von Hippel Lindau (pVHL) under normoxic conditions (58). pVHL is part of a multiprotein complex possessing associated E3 ubiquitin-ligase activity and is thought to target the degradation of HIF-1α (58, 59). Phosphorylation of Ser582, Ser583, or Ser584 by p38 may disrupt or prevent the formation of the HIF-1α-pVHL complex. It is, however, questionable whether HIF-1α is a direct target for p38 in sodium arsenite-treated OVCAR-3 cells. Activation of p38 already occurs after 1 h of sodium arsenite treatment, whereas HIF-1α protein accumulation is not detectable until 4 h (34). This suggests that p38 may regulate the HIF-1α protein indirectly, through phosphorylation of other factors. A possible candidate is PKF506, a binding protein-rapamycin-associated protein (FRAP), an effector of Akt, which may be activated by sodium arsenite in a p38-dependent manner (60). FRAP has been suggested to mediate hypoxia- and growth factor-induced HIF-1α protein expression (6).

The finding that up-regulation of HIF-1α protein as well as VEGF mRNA by arsenite is blocked by p38 kinase inhibitors would support that induction of VEGF expression is mediated by HIF-1. Elevation of VEGF mRNA expression in sodium arsenite-treated OVCAR-3 cells, however, was detected at earlier time points (after 2 h) than HIF-1α protein stabilization (after 4 h) (34). Moreover, exposure of OVCAR-3 cells to sodium arsenite did not result in transcriptional activation of a 5xHRE-dependent reporter gene in a transient transfection assay. Furthermore, treatment of OVCAR-3 with low concentrations of arsenic trioxide also up-regulated VEGF mRNA expression in thermore, treatment of OVCAR-3 with low concentrations of arsenite did not result in transcriptional activation of a 5xHRE-(after 4 h) (34). Moreover, exposure of OVCAR-3 cells to sodium arsenite treatment is even slightly higher in HIF-1α/H9251 cells than in HIF-1α/H18528 treated OVCAR-3 cells. Activation of p38 already occurs after several other transcription factors that can mediate transcriptional activation of VEGF in a HIF-1-independent manner under normoxic conditions (63, 64). As a first attempt to identify the transcription factors that mediate sodium arsenite-induced VEGF expression in OVCAR-3 cells, we performed transient transfection assays with luciferase reporter constructs containing the full-length VEGF promoter (–2274/+379) (11). Although the expression of this reporter construct was inducible by hypoxia in OVCAR-3 cells, we have never observed any activation of this reporter by sodium arsenite. Additional experiments are required to establish whether the effect of sodium arsenite on VEGF expression is the result of transcriptional activation or of mRNA stabilization. Further investigation of the mechanism that mediates sodium arsenite-induced VEGF expression and of the exact role of p38 in this effect will provide important knowledge on the regulation of VEGF expression under conditions of oxidative stress.

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