Recent evidence suggests that vascular endothelial growth factor (VEGF) expression is up-regulated by oxidative stressors through activation of hypoxia-inducible Factor 1 (HIF-1). To investigate whether this is a general phenomenon, we studied the effects of the sulfhydryl reagent arsenite on VEGF expression in human ovarian cancer cells. Arsenite potently induces the production of reactive oxygen species (ROS) systems and directly interacts with sulfhydryl groups of cellular thiols. We report that arsenite induces VEGF mRNA and protein levels in normoxic H134 and OVCAR-3 cells. Arsenite also increases HIF-1α protein levels, suggesting a role for HIF-1 in the induction of VEGF expression. Pretreatment with the ROS inhibitors catalase and mannitol attenuates arsenite-induced ROS production, but did not affect induction of VEGF mRNA and HIF-1α protein. In contrast, pretreatment with the thiol antioxidants glutathione or N-acetylcysteine completely abrogated both effects, whereas a potentiation was observed by depletion of intracellular glutathione. These results demonstrate that arsenite-induced VEGF mRNA and HIF-1α protein expression is independent of increased ROS production but critically regulated by the cellular reduced glutathione content. In addition, these data suggest the involvement of a thiol-sensitive mechanism in the regulation of VEGF mRNA expression and HIF-1α protein in human ovarian cancer cells.

Vascular endothelial growth factor (VEGF) is an important regulator of the process of angiogenesis in many types of cancer, including ovarian cancer. High VEGF expression and microvessel density have been correlated with poor survival in ovarian cancer patients. VEGF exists as multiple isoforms that can be generated by alternative splicing of a single transcript. VEGF₁₂₅ is efficiently secreted, whereas VEGF₁₆₅ is partially secreted and partly retained on the cell surface. Other variants, such as VEGF₁₄₅, VEGF₁₈₉, and VEGF₂₀₆, remain primarily associated to the cell surface and to the extracellular matrix. Expression of VEGF₁₂₅, VEGF₁₄₅, VEGF₁₆₅, and VEGF₁₈₉ mRNAs has been detected in human ovarian cancer cell lines (3, 4).

Although the VEGF gene is controlled by different transcription factors, the major regulator of its expression is hypoxia-inducible factor 1 (HIF-1). HIF-1 is composed of two subunits, HIF-1α and HIF-1β, that bind as a dimer to the hypoxia-responsive element (HRE) in the VEGF promoter (5). HIF-1 mediates activation of VEGF transcription in response to hypoxia in solid tumors and in various malignant cell lines (5, 6). Multiple stimuli, such as growth factors, hormones, nitric oxide, transition metals, and iron chelators, can induce VEGF expression in a HIF-1-dependent manner in normoxic cells (7–11).

The expression level of the HIF-1α protein is an important determinant for the activity of HIF-1. Although HIF-1β protein expression is detected in the nucleus of normoxic cells, HIF-1α protein is undetectable in most cell types due to rapid degradation by the ubiquitin-proteasome system (12–14). Hypoxia and many other activators of HIF-1 induce the accumulation of HIF-1α protein (9–11, 15). In hypoxic cells and in normoxic cells treated with transition metals or iron chelators, HIF-1α protein levels are elevated as a result of decreased ubiquitination and degradation (15). In some cases, induction of HIF-1α protein expression may also involve an increase in the rate of HIF-1α protein synthesis (16). In addition to its level of expression, the HIF-1α protein is regulated at the level of nuclear localization (17) and transactivation (15).

Recent studies suggest that alterations in the levels of reactive oxygen species (ROS) provide a redox signal for HIF-1 induction by hypoxia (18–20). Interestingly, ROS also appear to regulate HIF-1 activity under normoxia. In some cell types, increased ROS production has been shown to mediate HIF-1α protein accumulation and HIF-1-dependent transcription by hormones, growth factors, and transition metals (11, 19, 20). Moreover, direct exposure of cells to ROS may also induce HIF-1α protein levels and/or VEGF expression (20, 21).

Up till now, it is not established whether modulation of VEGF expression in tumor cells by ROS is a general phenomenon. If this would be the case, cytotoxic agents in use for cancer treatment might also up-regulate VEGF and, as a consequence, VEGF downstream events (22, 23). Recent evidence indicates that compounds containing trivalent arsenic (As³⁺, arsenite) have potential as therapeutic agents in cancer (24). Exposure to low dosages of arsenite in the form of arsenic trioxide (As₂O₃) and/or sodium arsenite (NaAsO₂) has a signif-

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; ROS, reactive oxygen species; HRE, hypoxia-responsive element; As³⁺, arsenic/arsenite; GSH, reduced glutathione; NAC, N-acetylcysteine; BSO, buthionine-sulfoximate; DCFH-DA, 2,7'-dichlorodihydrofluorescein diacetate; DCFH, 2,7'-dichlorofluorescein; DCF, dichlorofluorescein; pVHL, tumor suppressor protein von Hippel Lindau; MAPK, mitogen-activated protein kinase; nt, nuclotide(s); ELISA, enzyme-linked immunosorbert assay; SH, thiol group.
icant cytotoxic effect on different malignant cell lines (24). Arsenic compounds are potent inducers of oxidative stress, and evidence is provided that ROS may be mediators of arsenite-induced cytotoxicity (25, 26).

To analyze whether oxidative stress can influence VEGF expression in human ovarian cancer cells, we studied the effect of sodium arsenite (Na2AsO3). This agent has been shown to potently induce ROS production in several cell systems (25, 26). Increased ROS production may result from the activation of ROS-producing enzymes (25, 27) but may also be associated with depletion of reduced glutathione (GSH). Reduction of GSH levels by arsenite can be caused by the inhibition of glutathione reductase (28). In addition, arsenite directly interacts with thiol groups (SH) of GSH and cellular proteins (29–31).

Here, we report that arsenite induces VEGF expression in the human ovarian cancer cell lines OVCAR-3 and H134. Arsenite-increased VEGF expression was associated with the accumulation of HIF-1α protein in both cell lines, suggesting a role for HIF-1 in this effect. By using ROS inhibitors and thiol (anti)oxidants, we have assessed the possible involvement of increased ROS production, GSH depletion, or direct binding to thiol groups of cellular proteins in arsenite-induced VEGF expression and HIF-1α protein accumulation.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Sodium arsenite, actinomycin D, cycloheximide, mannitol, catalase, GSH, N-acetylcyesteine (NAC), and buthionine-sulfoximine (BSO) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands).

**Cell Culture and Cell Treatment—**The human ovarian cancer cell lines OVCAR-3 and H134 were cultured in Dulbecco’s modified Eagle’s medium (Dulbecco modified Eagle’s medium, Life Technologies, Inc., Breda, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum and 0.025% Tween 20)/5% milk and grown overnight. Thereafter, arsenite (100 or 30 μM) was added to the conditioned media, and cells were further incubated with depletion of reduced glutathione (GSH). Reduction of GSH levels by arsenite can be caused by the inhibition of glutathione reductase (28). In addition, arsenite directly interacts with thiol groups (SH) of GSH and cellular proteins (29–31).

We report that arsenite induces VEGF expression in the human ovarian cancer cell lines OVCAR-3 and H134. Arsenite-increased VEGF expression was associated with the accumulation of HIF-1α protein in both cell lines, suggesting a role for HIF-1 in this effect. By using ROS inhibitors and thiol (anti)oxidants, we have assessed the possible involvement of increased ROS production, GSH depletion, or direct binding to thiol groups of cellular proteins in arsenite-induced VEGF expression and HIF-1α protein accumulation.

**Western Blot Analysis—**In the Western blot experiments, 100 μg of protein cell extract was resolved in a SDS-polyacrylamide gel (7.5%) and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Etten-Leur, The Netherlands). Membranes were blocked for 1 h in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and then incubated overnight at 4 °C with the mouse monoclonal antibody to HIF-1α (H1alpha67, catalogue no. ab1-100) and the horseradish peroxidase-directed antiserum in a 1:1000 dilution. After washing with TBST, the membranes were incubated for 1 h with horseradish peroxidase-linked anti-mouse antiserum in TBST/5% milk. The membranes were washed again with TBST, and proteins were visualized by Electro Chemiluminescence. The mouse monoclonal antiserum to HIF-1α (H1alpha67, catalogue no. ab1-100) and the horseradish peroxidase-coupled anti-mouse serum (catalogue no. P0260) were from Novus Biologicals/AbCam (Cambridge, United Kingdom) and DAKO (Glostrup, Denmark), respectively.

**Measurement of ROS—**Intracellular ROS production was assessed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Leiden, The Netherlands). After diffusion into cells, this dye is hydrolyzed by intracellular esterase to yield 2′,7′-dichlorofluorescein (DCF). ROS (hydrogen peroxide or low molecular weight peroxides) in the cells oxidizes DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Cells were plated on culture dishes in medium and grown overnight. Thereafter, arsenite (100 μM) was added to one volume at a concentration of 100 μM. Subsequently, conditioned media with or without arsenite was again added to eight culture dishes with cells. Thus, at T = 0 the amount of VEGF protein in the medium was the same in each culture dish. After incubation periods of 2, 4, 6, and 8 h, 450 μl of conditioned medium was sampled and cells were lysed. VEGF concentrations in nondiluted media samples and lysates were determined in duplicate by ELISA using the reagents and the protocol supplied with the Quantikine Human VEGF Immunoassay kit (R&D Systems/ITK Diagnostics, Uithoorn, The Netherlands). Differences in VEGF concentrations in medium and lysates of nontreated versus arsenite-treated cells were evaluated using Student’s t test for two groups. p values <0.05 were considered to be significant.

**Measurement of ROS—**Intracellular ROS production was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Leiden, The Netherlands). After diffusion into cells, this dye is hydrolyzed by intracellular esterase to yield 2',7'-dichlorofluorescein (DCF). ROS (hydrogen peroxide or low molecular weight peroxides) in the cells oxidizes DCFH to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF). Cells were plated on culture dishes and incubated with arsenite (100 μM) for different time periods. DCFH-DA was added to the medium 1 h before harvesting the cells by trypsinization. Cell fluorescence was detected with a FACScan flow cytometer using a 525-nm band pass filter. The relative mean fluorescence intensity in arsenite-treated cells after individual incubation periods (i) was tested against 100% (t = Mi) – 100/S.E.) by means of Student’s t test for one group. The p values <0.05 were considered to be significant. In addition, the relative mean fluorescence intensity in cells treated with arsenite and catalase or mannitol (c, m) was tested against the relative mean fluorescence intensity in cells treated with arsenite alone or to control cells (t = Mc, m) – 100/S.E.) using the Student’s t test for two groups and one group, respectively. The p values <0.05 were considered significant.
Arsenite Induces VEGF mRNA and Protein Levels in OVCAR-3 and H134 Cells—VEGF mRNA levels in the human ovarian cancer cells OVCAR-3 and H134 upon different periods of exposure to 100 μM arsenite were assessed by the RNase protection assay (Fig. 1). In this assay, a labeled, antisense VEGF<sub>165</sub> RNA probe was used for hybridization, allowing efficient detection of mRNAs encoding the VEGF<sub>165</sub> isoform. Hybridization of total RNA of nontreated OVCAR-3 and H134 cells resulted in the protection of a 252-nt fragment, indicating a basal level of expression of VEGF<sub>165</sub> in both cell lines. Judged by the intensity of the 252-nt protected fragment, the basal level of VEGF<sub>165</sub> mRNA in H134 cells appeared to be slightly higher than that in OVCAR-3 cells. After 2 h of arsenite treatment, the intensity of the 252-nt fragment relative to the γ-actin-protected fragment increased in both OVCAR-3 and H134 cells, indicating an increase in VEGF<sub>165</sub> mRNA levels. This increase was sustained until at least 8 h of exposure. At this time point, induction of VEGF<sub>165</sub> mRNA levels was ~6- and 3-fold in OVCAR-3 and H134 cells, respectively. Changes in VEGF<sub>165</sub> mRNA levels were not observed in nontreated cells (data not shown).

We next assessed whether induction of VEGF mRNA levels by arsenite resulted in an increased production of VEGF protein. Arsenite was added to the conditioned medium of OVCAR-3 and H134 cells, and VEGF concentrations were measured by ELISA in the conditioned media or in cell lysates after different periods of incubation. In addition, we determined VEGF protein concentrations in conditioned media and lysates of nontreated cells. As can be seen in Fig. 2A, VEGF protein levels in the conditioned medium of H134 cells were much higher than in those of OVCAR-3 cells at the start of treatment (T = 0). Upon exposure to arsenite, a time-dependent increase in VEGF production was observed in both cell types. Increased VEGF protein levels in the conditioned media of arsenite-treated versus nontreated cells was observed after 6 and 8 h of incubation. In OVCAR-3 cells, this increase was statistically significant. In the lysates, increased production of VEGF was evident and statistically significant after 4 h of incubation and was more pronounced after 6 and 8 h (Fig. 2B).

It should be noted that, in contrast to the RNA protection assay, the ELISA assay does not discriminate between VEGF<sub>165</sub> and the other VEGF isoforms. Therefore, it is difficult to assess exact correlations between VEGF mRNA and protein levels. Nevertheless, these data indicate that induction of VEGF mRNA upon arsenite treatment is associated with the accumulation of VEGF protein in H134 and OVCAR-3 cells and in their conditioned media.

Arsenite-induced VEGF Expression Is at the Transcriptional Level and Is Dependent on de Novo Protein Synthesis—To determine whether induction of VEGF mRNA levels by arsenite was due to increased transcription or to RNA stabilization, we analyzed the effect of the transcription inhibitor actinomycin D in both OVCAR-3 and H134 cells. Fig. 3 shows that actinomycin D completely inhibited induction of VEGF<sub>165</sub> mRNA levels by arsenite in both cell types, suggesting that this effect is at the transcriptional level.

To analyze whether arsenite-induced expression of VEGF<sub>165</sub> was dependent on de novo protein synthesis, we determined the effect of the protein synthesis inhibitor cycloheximide. As can be seen in Fig. 4A, induction of VEGF<sub>165</sub> mRNA levels upon arsenite treatment in OVCAR-3 cells was strongly inhibited by cycloheximide. In H134 cells, VEGF<sub>165</sub> mRNA levels were potentiated with cycloheximide alone. Incubation of cycloheximide-treated H134 cells with arsenite did, however, not increase VEGF<sub>165</sub> mRNA levels beyond those observed by cycloheximide alone. These data suggest that induction of VEGF expression by arsenite is dependent on ongoing protein synthesis.

Arsenite-induced VEGF Expression Is Associated with the Accumulation of HIF-1α Protein—To examine whether arsenite-induced VEGF expression may be mediated by HIF-1, we investigated the HIF-1α protein levels upon arsenite treatment in OVCAR-3 and H134 cells by Western blot with a HIF1α-directed antibody expression. As can be seen in Figs. 4B and 5A, OVCAR-3 and H134 cells showed a basal level of HIF-1α protein expression. After 4 h of arsenite treatment, the level of HIF-1α protein was elevated and was even further increased after 8 h. Note that, under conditions where cycloheximide was found to inhibit arsenite-induced VEGF mRNA levels, the accumulation of HIF-1α protein was inhibited over 90% in both OVCAR-3 and H134 cells (Fig. 4B).

We also assessed the effects of arsenite on the levels of HIF-1α mRNA by the RNase protection assay (Fig. 5B). Hybridization of total RNA of nontreated OVCAR-3 cells to a HIF-1α antisense probe resulted in the protection of a fragment with the expected size of 119 nt. As a positive control, we also hybridized total RNA of OVCAR-3 cells that were transiently transfected with a HIF-1α expression vector. As expected, the 119-nt fragment was detected with increased intensity, whereas it was not observed after hybridization with control tRNA. These data confirm that the 119-nt fragment is protected by HIF-1α mRNA and that detection of this fragment is indicative for a basal level of expression of HIF-1α mRNA in nontreated OVCAR-3 cells. The intensity of the 119-nt fragment did not alter relatively to that of the γ-actin-protected fragment until at least 8 h of exposure to arsenite. The same results were obtained in H134 cells (data not shown). These findings indicate that HIF-1α mRNA levels are not influenced upon treatment with arsenite and that induction of HIF-1α...
protein is regulated by a post-transcriptional mechanism.

The low level of expression in nonstimulated OVCAR-3 and H134 cells suggests that HIF-1α protein is unstable under normoxic conditions in these cell lines. To assess whether arsenite regulates the expression of HIF-1α protein by inhibiting its degradation, we monitored the levels of arsenite-induced HIF-1α protein after blocking protein synthesis by cycloheximide. First, we confirmed that HIF-1α protein is rapidly degraded in the absence of protein synthesis in nonstimulated OVCAR-3 and H134 cells under normoxia. To this end, we

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**Fig. 2. Sodium arsenite induces VEGF protein levels in OVCAR-3 and H134 cells.** Sodium arsenite (100 μM) was added to conditioned media of OVCAR-3 and H134 cells and at the indicated time points the concentrations of VEGF protein in conditioned media (A) and cell lysates (B) were measured by ELISA as described under “Experimental Procedures.” As a control, VEGF protein concentrations were determined in conditioned medium and lysates of nontreated cells. Results are given in picograms of VEGF per milliliter of conditioned medium in (A) and per milligram of total cell protein in (B). The histograms represent the mean ± S.D. of duplicate samples in a representative experiment of three independent experiments that gave comparable results. Significant differences in mean VEGF concentrations in medium and lysates of nontreated versus arsenite-treated cells after individual incubation periods are indicated by an asterisk (p < 0.05).

**Fig. 3. Induction of VEGF<sub>165</sub> mRNA by sodium arsenite is at the transcriptional level.** OVCAR-3 and H134 cells were exposed for the indicated time periods to sodium arsenite (100 μM) in the absence or presence of actinomycin D (5 μg/ml) or to actinomycin D (5 μg/ml) alone. Actinomycin D was added 30 min prior to the addition of sodium arsenite. Total RNA was extracted and hybridized to VEGF<sub>165</sub> and γ-actin antisense probes in the RNase protection. rRNA (T) was hybridized as a negative control. The 252- and 130-nt fragments protected by the mRNAs of VEGF<sub>165</sub> and γ-actin and the full-length probes are indicated.
investigated the decay of hypoxia-stabilized HIF-1α after transfer of cells from hypoxia to normoxia. In agreement with previous studies in other cell types (12, 13, 35), a rapid decay of HIF-1α protein was observed within 15 min after transfer from hypoxia to normoxia in OVCAR-3 and H134 cells (Fig. 5C and data not shown). HIF-1α protein was completely undetectable by the end of 30 min under normoxia. In contrast, arsenite-induced HIF-1α protein levels under normoxia remained constant for 15 min after cycloheximide addition and decreased relatively slowly to 50% within 45 min thereafter. Despite the lack of protein synthesis, the levels of HIF-1α protein were still detectable and clearly above basal after 3 h of cycloheximide exposure. The decay of arsenite-induced HIF-1α protein in the absence of protein synthesis was found to occur with similar kinetics in H134 cells (data not shown). Note that the effects of arsenite and hypoxia in OVCAR-3 cells were specific for HIF-1α protein, because the levels of a protein that was nonspecifically recognized by the HIF-1α or secondary antisera remained constant under all conditions. These results indicate that arsenite induces HIF-1α protein in normoxic OVCAR-3 and H134 cells by slowing down its degradation.

The finding that increased levels of VEGF165 mRNA are associated with the stabilization of HIF-1α protein in arsenite-treated OVCAR-3 and H134 cells may be indicative for a role of HIF-1 in the induction of VEGF expression by arsenite.

Arsenite-induced VEGF Expression and HIF-1α Protein Accumulation Is Independent of ROS—Arsenite has been reported to induce the production of different types of ROS, including superoxide anions (O2•−), hydrogen peroxide (H2O2), and hydroxyl radicals (OH•) (26). To investigate the role of ROS in arsenite-induced VEGF expression and HIF-1α protein accumulation, we first tested whether arsenite treatment increased the production of H2O2 in OVCAR-3 cells. OVCAR-3 cells were treated with 100 μM arsenite for different time periods, and 80 μM DCFH-DA was added to the medium 1 h before cells were harvested by trypsinization. Intracellular peroxide levels in trypsinized cells were monitored by flow-cytometric analysis. Fig. 6 shows that a gradual increase in intracellular peroxide levels was detectable at the various time points tested, which reached statistical significance after 8 h of exposure.

To further investigate a possible relationship between the induction of VEGF expression and HIF-1α protein and intracellular peroxide levels in arsenite-treated OVCAR-3 cells, we tested the effect of the ROS inhibitors catalase and mannitol. Catalase can inactivate hydrogen peroxide by decomposing it into water and oxygen, whereas mannitol is a selective scavenger of hydroxyl radicals. Because hydrogen peroxide can

![Graph showing VEGF and HIF-1α protein levels](image_url)
Fig. 5. Sodium arsenite induces the stabilization of HIF-1α protein. A and B, OVCAR-3 cells were exposed to sodium arsenite (100 μM) and protein, and total RNA was extracted at the indicated time points. HIF-1α protein and mRNA levels were assessed by Western blotting with a HIF-1α-directed antiserum (A) and the RNase protection assay (B), respectively. In the RNase protection experiment, total RNA was hybridized to a HIF-1α antisense RNA probe. As a positive control, hybridization was performed with total RNA of OVCAR-3 cells that were transiently transfected with an expression vector encoding human HIF-1α (PC). tRNA (T) was hybridized as a negative control. The 119- and 130-nt fragments protected by the mRNAs of HIF-1α and γ-actin and the full-length probes are indicated. C, HIF-1α expression was induced by exposure of OVCAR-3 cells to 1% O2 (hypoxia (Hyp); upper panel) for 6 h or to sodium arsenite (100 μM) for 5 h under normoxia (Norm, lower panel). Cycloheximide (CHX) was added to a final concentration of 100 μM, and cells were further incubated under normoxia. Cell lysates were prepared at the indicated time periods after cycloheximide addition. HIF-1α protein levels were assessed by Western blotting with a secondary horseradish peroxidase-linked antiserum in A and C, an unidentified protein that is specifically recognized by the HIF-1α-directed antiserum or by the secondary horseradish peroxidase-linked antiserum is indicated as NS.

HIF-1α protein accumulation (Fig. 7A), pretreatment with mannitol (50 or 100 mM) or catalase (500 or 1000 units/ml) was found to significantly reduce arsenite-induced fluorescence intensity to levels that were not significantly below those observed in control cells. This indicates that induction of hydrogen peroxide levels by arsenite was efficiently blocked. In contrast, induction of VEGF165 mRNA and HIF-1α protein levels was hardly affected by mannitol and catalase pretreatment (Fig. 7, B and C). In the experiment presented in Fig. 7A, 9 h of exposure to mannitol (100 mM) or catalase (1000 units/ml) alone was also found to reduce the basal fluorescence intensity of 40.5 (control, M1) to 29.1 and 9.9, respectively (histograms not shown). Both agents, however, did not significantly influence the basal levels of VEGF165 mRNA and HIF-1α protein in OVCAR-3 cells. The concentrations of mannitol and catalase that were used here were not toxic for OVCAR-3 cells and have been shown to be nontoxic in other in vitro studies. All together, these results strongly suggest that arsenite-induced VEGF expression and HIF-1α protein accumulation is independent of increased ROS production in OVCAR-3 cells.

Induction of VEGF Expression and HIF-1α Protein by Arsenite Is Modulated by Intracellular GSH Levels in OVCAR-3 Cells—Previous studies have shown that the level of intracellular GSH is an important determinant for the biological effects of arsenite. Elevated levels of intracellular GSH often antagonize the effects of arsenite, whereas they are potentiated by depletion of intracellular GSH (28, 36, 37). To examine the role of intracellular GSH levels in the induction of VEGF mRNA levels and HIF-1α protein by arsenite in OVCAR-3 cells, we tested the effect of pretreatment with GSH and NAC. The latter agent is known to induce intracellular GSH levels by acting as a precursor for the synthesis of GSH. As shown in Fig. 8A, pretreatment with GSH inhibited induction of VEGF165 mRNA levels by arsenite in a concentration-dependent manner. A partial inhibition was observed with 10 mM GSH, whereas complete inhibition was obtained at the 20 mM concentration. Induction of VEGF165 mRNA levels was also completely abrogated by pretreatment with 10 and 20 mM concentrations of NAC. The inhibitory effect of GSH and NAC on VEGF165 mRNA induction was associated with the inhibition of HIF-1α protein accumulation (Fig. 8B). By themselves, GSH (20 mM) and NAC (20 mM) had only a minor effect on VEGF165 mRNA and HIF-1α protein levels in OVCAR-3 cells.

To obtain additional evidence for a role of GSH in arsenite-induced VEGF expression and HIF-1α protein accumulation in OVCAR-3 cells, we assessed the effect of pretreatment with BSO. BSO depletes the intracellular GSH pool by inhibiting glutamylcysteine synthase, which plays a critical role in the synthesis of GSH (38). Because arsenite is known to reduce GSH levels (28), we speculated that potential effects of GSH depletion would be more pronounced when cells were treated with a suboptimal dose of arsenite (30 μM). As can be seen in Fig. 9A, OVCAR-3 cells displayed a slower, more gradual increase in VEGF165 mRNA and HIF-1α protein levels upon treatment with 30 μM arsenite. Pretreatment with BSO (500 μM) was found to potentiate the elevation of VEGF165 mRNA as well as HIF-1α protein levels after 6 and 8 h of exposure to
arsenite (Fig. 9). Pretreatment with BSO alone (500 µM) for a period of 24 h hardly affected the basal levels of VEGF mRNA and HIF-1α protein in OVCAR-3 cells. In summary, these results suggest that the intracellular GSH content is an important determinant in the regulation of VEGF expression and HIF-1α protein levels by arsenite in OVCAR-3 cells.

**DISCUSSION**

This study was undertaken to examine whether oxidative stress can influence VEGF expression levels in human ovarian cancer cells. To this end, we examined the effects of the oxidative stressor arsenite in the two human ovarian cancer cell lines OVCAR-3 and H134. Arsenite was found to induce VEGF mRNA and VEGF protein levels in both cell lines. Our results suggest that this effect involves transcriptional activation of the VEGF gene. First, elevation of VEGF mRNA levels in arsenite-treated OVCAR-3 and H134 cells was completely attenuated by the transcription inhibitor actinomycin D. Second, induction of VEGF mRNA levels was found to be associated with the accumulation of HIF-1α protein in both cell lines, indicating that arsenite may induce VEGF expression through the activation of HIF-1. This would be consistent with our results from experiments with cycloheximide showing that arsenite-induced VEGF expression is dependent on de novo protein synthesis.

It should be noted that, in both cell lines, an increase in VEGF mRNA levels was detectable after 2 h of exposure to 100 µM arsenite, whereas clear elevation of HIF-1α protein levels was not detected until 4 h of exposure (Figs. 1 and 5). Consistently, OVCAR-3 cells that were treated with a suboptimal dose of arsenite (30 µM) displayed increased VEGF mRNA levels after 4 h of treatment, whereas induction of HIF-1α protein was observed after 6 h (Fig. 9). Because a basal level of HIF-1α protein expression is detected in both cell lines, arsenite may increase HIF-1 activity at earlier time points by affecting the nuclear localization or the transcriptional activity of pre-existing HIF-1α protein. Alternatively, arsenite-induced VEGF expression may involve the activation of other or additional transcription factors. A possible candidate may be the transcription factor AP-1, which has been shown to be activated by arsenite (37). The VEGF promoter contains six potential binding sites for AP-1 that are located at positions –1892 to –1886, –1227 to –1221, –1129 to –1123, –937 to –931, –490 to –484, and +418 to +424 relative to the transcription start at position +1 (5, 39, 40). Deletion/mutation analysis of the VEGF promoter using luciferase-reporter constructs has implicated AP-1 transcription factors in VEGF transcription regulation. AP-1 binding to –1129 to –1123 and to –937 to –931 potentiates HIF-1-mediated induction of VEGF expression by hypoxia in human glioma cell lines (10, 40). In one of those cell lines, the AP-1 element at –937 to –931 was also shown to potentiate HIF-1-dependent activation of VEGF by nitric oxide (10). AP-1 may, however, also mediate induction of VEGF expression in an HIF-1-independent manner (41, 42). Additional transcription factors that are involved in VEGF regulation are SP-1 and AP-2, which mediate activation of VEGF by various growth factors (43–46). Thus far, it has hardly been explored whether arsenite can affect SP-1 and AP-2 activity. Additional experiments should be performed to establish whether arsenite induces functional HIF-1 and whether HIF-1 contributes to arsenite-induced VEGF expression.

In both OVCAR-3 and H134 cells, HIF-1α mRNA levels remained unaltered upon arsenite treatment, indicating that induction of HIF-1α protein is regulated on the protein level.
Many activators of HIF-1, including hypoxia, have been shown to induce HIF-1α protein expression by inhibiting ubiquitination and degradation (15). To investigate whether arsenite induces HIF-1α protein accumulation through a similar mechanism in OVCAR-3 and H134 cells, we assessed the kinetics of the decay of arsenite-induced HIF-1α protein in the absence of protein synthesis. As a reference for the stability of HIF-1α under normoxia in nontreated OVCAR-3 and H134 cells, we investigated the decay of hypoxia-stabilized HIF-1α protein upon reoxygenation in the same manner. The decay of arsenite-induced HIF-1α protein was found to be significantly slower in comparison with the very rapid decay of hypoxia-stabilized HIF-1α protein observed upon reoxygenation. In agreement with findings in other cell types (12, 13, 35), these results confirm that HIF-1α protein is unstable in normoxic OVCAR-3 and H134 cells and suggest that arsenite induces the accumu-
lation of HIF-1α protein by inhibiting its degradation.

As increased ROS production has been suggested to mediate the stabilization of HIF-1α (11, 19, 20), we assessed the role of ROS in arsenite-induced HIF-1α protein and VEGF expression. By using DCFH-DA, we demonstrated that arsenite increased the production of hydrogen peroxide in a time-dependent manner in OVCAR-3 cells. Pretreatment with catalase or with the hydroxyl radical scavenger mannitol efficiently attenuated arsenite-induced hydrogen peroxide production, but failed to suppress elevation of HIF-1α protein and VEGF mRNA levels. These results strongly suggest that both effects of arsenite are independent of increased ROS production.

The role of ROS in the regulation of HIF-1α and/or VEGF is controversial. By investigating cells lacking mitochondrial DNA, Chandel et al. (20) have provided evidence that increased production of mitochondrial ROS is required for stabilization of HIF-1α protein in response to hypoxia. In a recent study in two different cell types that were also depleted of mitochondrial DNA, HIF-1 activation by hypoxia was unaffected (47). Another model proposes that the response to hypoxia may be caused by a decreased production of ROS by NADPH oxidase (18). In this model, decreased production of ROS is postulated to have an inhibitory effect on HIF-1α protein degradation. In line with this hypothesis, Haddad et al. (48) have demonstrated that antioxidants prevent the decay of HIF-1α protein upon reoxygenation. The role of ROS in the regulation of HIF-1α and
VEGF in normoxic cells is also not well established. Results from Chandel et al. (19) have indicated that HIF-1α protein stabilization and HIF-1-dependent transcription in response to the transition metal cobalt (cobalt chloride) were mediated by increased production of cytoplasmic ROS. In contrast, inhibition of cobalt chloride-induced ROS production was found not to influence HIF-1-dependent transcription in a study of Salnikow et al. (49). The reason for these controversial results is not clear, but may be attributed to differences between the investigated cell types. In fact, ROS-mediated induction of HIF-1 by growth factors and hormones under normoxia also seems to be a cell type-specific effect (11). Thus, some reports, including our own results obtained with arsenite, suggest that elevated levels of ROS do not trigger HIF-1 activation per se.

Part of the biological effects of arsenite are likely to be caused by its ability to lower intracellular GSH levels and by its binding to free thiol (SH) groups of critical cellular proteins (28). Consistently, elevation of GSH levels is found to antagonize the effects of arsenite (36, 37). In agreement with these studies, we demonstrated that intracellular GSH levels also modulate the effects of arsenite on HIF-1α protein and VEGF expression in OVCAR-3 cells. Pretreatment with GSH or NAC (a precursor for GSH) completely attenuated induction of HIF-1α protein and VEGF mRNA levels, whereas upon depletion of intracellular GSH by BSO the reverse was shown. Thus, elevated levels of intracellular GSH inhibit arsenite-induced HIF-1α protein and VEGF mRNA levels.

The exact mechanism by which intracellular GSH antagonizes induction of HIF-1α protein and VEGF expression remains to be determined. It is unlikely that induction of HIF-1α protein and VEGF expression by arsenite is simply mediated by GSH depletion. As demonstrated, suppression of intracellular GSH by BSO pretreatment did not influence HIF-1α protein and VEGF mRNA levels in OVCAR-3 cells. It is more conceivable that induction of VEGF expression and HIF-1α protein by arsenite involves its binding to thiol groups of cellular proteins. GSH may antagonize both effects of arsenite by competitive interference with the interaction between arsenite and the thiol groups of cellular proteins (29, 30). High levels of GSH may thus prevent arsenite from binding to these proteins.

It is of interest to identify the cellular proteins whose interaction with arsenite results in the induction of HIF-1α protein and VEGF expression. A possible candidate may be the HIF-1α protein itself. The degradation of HIF-1α under normoxic conditions is thought to be targeted by the tumor suppressor protein von Hippel Lindau (pVHL) (50, 51). pVHL is part of a multiprotein complex possessing associated E3 ubiquitin-ligase activity (51). In normoxic cells, HIF-1α protein exists in a complex with VHL. In hypoxic cells and in normoxic cells treated with cobalt chloride or with the iron chelator desferrioxamine, the VHL-HIF-1α protein complex is dissociated, which prevents the degradation of HIF-1α protein (50, 52, 53). The interaction of HIF-1α with pVHL is mediated by a small domain in the HIF-1α protein called the oxygen-dependent-degradation domain (50). This domain contains an unpaired cysteine residue (54). Although arsenite is thought to have a much higher affinity for dithiols (vicinal thiols) than for monothiols (28), it is possible that arsenite disrupts the HIF-1α-VHL protein complex by binding to the thiol group of this cysteine residue.

Other potential targets for arsenite may be some of the enzymes that participate in the ubiquitin-proteasome system. Arsenite has been suggested to inhibit ubiquitin-dependent protein degradation at multiple steps through its binding with vicinal thiol groups of different components of this proteolytic pathway (55). Although this effect of arsenite has only been demonstrated in rabbit reticulocytes and in reticulocyte lysates (55), it cannot be excluded that arsenite induces HIF-1α protein accumulation by acting as a general inhibitor of the ubiquitin-proteasome system. General proteasome inhibitors have indeed been shown to induce HIF-1α protein accumulation under normoxic conditions. These agents appear, however, incapable of inducing functional HIF-1 because they were found not to stimulate HIF-1-dependent transcription (14). The fact that arsenite induces VEGF expression may indicate that arsenite influences additional steps in the regulation of HIF-1α or that this agent regulates HIF-1α protein through a different mechanism.

Another mediator of arsenite-induced HIF-1α protein stabilization and VEGF expression may be nitric oxide, the production of which is increased in some cell types upon arsenite treatment (56). Interestingly, nitric oxide also reacts with thiol groups of cellular proteins, and nitrosylation of thiol residues has been suggested to play a role in nitric oxide-induced stabilization of HIF-1α protein under normoxic conditions (57). Inhibitors of cellular enzymes that synthesize nitric oxide (nitric-oxide synthase inhibitors) do, however, not significantly affect arsenite-induced HIF-1α protein accumulation and VEGF expression in OVCAR-3 cells (data not shown).

In addition to ROS and pVHL, the one or more signaling pathways that regulate HIF-1 activity contain several other intermediates. Inhibitors of phosphatidylinositol 3-kinase and of the small GTPase Rac-1 can block HIF-1α protein stabilization and transactivation by hypoxia. Inhibition of the mitogen-activated protein kinase (MAPK) family members p38 MAPK and p44/p42 MAPKs also has a negative effect on the transactivation function of HIF-1α in hypoxic cells (58). These factors have been implicated in the regulation of HIF-1 and/or VEGF expression in normoxic cells as well (58–61). Interestingly, arsenite can enhance the activity of phosphatidylinositol 3-kinase as well as of Rac1, p38 MAPK, and p44/p42 MAPKs (37, 62, 63). We are currently assessing a possible involvement of these components in the effects of arsenite on HIF-1α protein and VEGF expression.

It is interesting to note that our results with arsenite may also be of clinical relevance. Despite the fact that trivalent arsenic (As3+) is toxic to humans, arsenic trioxide (As2O3) appears to be a promising agent in the treatment of cancer when used at low dosages (24). As2O3 has been shown to promote apoptosis in endothelial cells and in several types of tumor cells. Based on the observation that low dosages of As2O3 (~10–6 M) have a significant cytotoxic effect on human ovarian cancer cell lines, it was suggested that As2O3 may also be a useful agent for the treatment of ovarian cancer (64). Although the effects of arsenite on VEGF expression and HIF-1α protein in OVCAR-3 and H134 cells were observed at relatively high concentrations (30 and 100 μM), our study may indicate that caution should be taken with regard to such an approach.

In this study, we show for the first time that HIF-1α protein and VEGF expression can be induced by a thiol-reactive agent in human ovarian cancer cells. These findings may have implications for cytotoxic agents in cancer treatment, such as highly reactive platinum compounds and alkylating agents, which are susceptible of interacting with thiol groups of cellular sulphydryls.

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