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Critical roles of AMP-activated protein kinase in the carcinogenic metal-induced expression of VEGF and HIF-1 proteins in DU145 prostate carcinoma

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Abbreviations:
ACC, acetyl-CoA carboxylase
AMPK, AMP-activated protein kinase
As, sodium arsenite
Co, cobalt chloride
DN, dominant negative
ELISA, enzyme-linked immunosorbent assay
ERK, extracellular-regulated kinase
GSH, reduced glutathione
HIF-1, hypoxia-inducible factor-1
JNK, c-jun-NH_2-terminal kinase
MAPK, mitogen-activated protein kinase

ABSTRACT

Epidemiological and experimental animal data indicate that exposure to both metals and metalloid species exacerbates the risk of human diseases, particularly cancers. Vascular endothelial growth factor (VEGF), which performs a primary function in both tumor progression and angiogenesis, is up-regulated due to exposure to an array of carcinogenic metals, but the mechanisms responsible for the metal activation remain somewhat poorly understood. Recently, we demonstrated that AMP-activated protein kinase (AMPK), which acts as an energy sensor, providing metabolic adaptation effects under ATP-deprived conditions, is critical for the expression of VEGF under oxygen- and glucose-deprived conditions. As carcinogenic metals are potent VEGF expression inducers, we hypothesized that AMPK would also play a crucial role in metal-induced VEGF expression. Here, we present evidence that carcinogenic metals such as arsenite, vanadate, and cobalt, induce AMPK activation and VEGF expression via several different mechanisms, and that AMPK is able to regulate the expression of VEGF mRNA in a hypoxia-inducible factor-1-dependent or -independent manner, depending on the metal applied. We also attempted to characterize the relevant signal transduction pathways in metal-induced VEGF expression and AMPK activation, as well as the role of reactive oxygen species within this context. Overall, our data suggest that AMPK is a critical regulatory component in metal-induced VEGF expression, which further implies its intrinsic involvement in metal-induced carcinogenesis.

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NAC, *N*-acetyl-cysteine
ROS, reactive oxygen species
RT, reverse transcriptase
Va, sodium orthovanadate
VEGF, vascular endothelial growth factor
WT, wild type

1. Introduction

Metals are essential life elements, and have been shown to regulate an array of biological and biochemical functions in every living cell [1]. However, overexposure to certain metals, as is known to occur in a variety of environmental and occupational settings, has been associated with the development of a variety of cancers [2]. In fact, current epidemiological and experimental data from animal studies have revealed that some metals and metal-containing compounds, including vanadium, chromium, arsenite, cobalt, and nickel, can exacerbate the risk of developing cancers of the prostate, kidney, bladder, and liver, as well as lymphoma and leukemia [3]. Such metals are globally distributed throughout our environment, due to their extensive use in modern society. Therefore, the exposure of humans to such metals is all but inevitable. Nevertheless, the exact mechanisms underlying the toxicity and carcinogenicity of these metals remain fairly poorly understood, although metal exposure has been demonstrated to activate a variety of cancer-related genes, such as VEGF, ras, c-src, and members of the family of mitogen-activated protein kinases (MAPK) [4].

VEGF is the most potent known mitogen for endothelial cells, and is a specific activator of angiogenesis in several cancer types, including prostate cancer [5]. Elevated VEGF levels have been observed in a variety of tumor cells [6]. Although the VEGF gene is controlled by many different transcription factors, hypoxia-inducible factor-1 (HIF-1) has been identified as the principal regulator. HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β, and its activity is regulated principally by the accumulation of HIF-1α protein; this subunit is degraded rapidly via the ubiquitin-proteasome system under normoxic conditions, but hypoxic conditions result in a blockage of HIF-1α degradation, thereby inducing its accumulation [7–9]. In addition to protein stabilization, HIF-1 regulation includes mechanisms at the level of transcription, post-translational modification, nuclear translocation, and association with transcriptional cofactors [10]. Hypoxia is a potent stimulus for increased VEGF and HIF-1 expression in rapidly growing solid tumors. However, a variety of stimuli, including cytokines, metal ions, oxidative stressors, and hormones, have been found to exacerbate the production of VEGF under normoxic conditions, in an HIF-1-dependent manner [11–16]. Among these stimuli, the mechanisms responsible for metal-induced VEGF expression remain to be most elusive.

AMPK, a heterotrimer which consists of a catalytic subunit (α) and two regulatory subunits (β and γ), performs a central function as an energy sensor, via the coordination of a number of adaptive responses under ATP-depleting metabolic stresses. AMPK can be sensitively activated via increases in the cellular AMP-to-ATP ratio as the result of ischemia/hypoxia, nutritional deprivation, oxidative stress, and exercise [17]. Once activated, AMPK shuts down ATP-consuming metabolic pathways, whereas AMPK facilitates the activation of energy producing pathways in order to protect the cells [18]. We have recently demonstrated that the activity of AMPK is critical for VEGF expression under oxygen- and glucose-deprived conditions in various cancer cells [19,20], thereby suggesting that the AMPK-mediated energy sensing signal is crucial in the adaptive responses of cancer cells.

As carcinogenic metals are potent VEGF expression inducers, we hypothesized that AMPK would also play a crucial role in metal-induced VEGF expression, and have attempted to test our hypothesis in the present study. Among the variety of carcinogenic metals and metalloids suggested by the epidemiological and experimental data to constitute pathologically or physiologically relevant stimuli for VEGF expression, we elected to study cobalt [21,22], vanadate [23], and arsenite [24], as these metals had been reported to elevate VEGF expression in a variety of cancer cells, via different mechanisms. We reasoned that determining the role of AMPK under multiple regulatory conditions for VEGF expression might help us to understand the nature of the energy sensing signal in a more collective and comprehensive way. Cobalt is a well-known hypoxia surrogate, which stabilizes HIF-1α by blocking its degradation under normoxic conditions, therefore enhancing VEGF transcription in a manner similar to that seen under hypoxic conditions [21,22]. In the case of vanadate, the phosphatidylinositol-3 kinase pathway was implicated in the induction of HIF-1α expression [23], and we recently demonstrated that vanadate also stimulate HIF-1α protein synthesis [25]. Arsenite has also been reported to induce the expression of VEGF in an HIF-1-independent manner [26]. However, the precise mechanism underlying these effects remains to be even preliminarily elucidated. Here, we demonstrate that these metals activate AMPK in cancer cells via different mechanisms and that AMPK is indeed crucial for the metal-induced VEGF expression. Moreover, we attempted to characterize the function of reactive oxygen species (ROS) as upstream signals, and also evaluated the role of the metal-induced signal pathway in this process.

2. Materials and methods

2.1. Materials

RPMI medium 1640 and fetal bovine serum were purchased from Life Technologies, Inc. Sodium arsenite, cobalt chloride, sodium orthovanadate, glutathione (GSH), *N*-acetylcysteine (NAC), and cycloheximide were obtained from Sigma. Catalase

was from ICN Biomedicals Inc. The anti phospho-specific antibodies that recognize a phosphorylated ACC-Ser^{79}, AMPKα-Thr^{172}, p38 MAPK (Thr^{180}/Tyr^{182}), Akt-Ser^{473}, and AMPKα antibody were from Cell Signaling Technology. Antibody for HIF-1α was obtained from BD Biosciences. Antibodies for HIF-1β, and c-myc were purchased from Santa Cruz Biotechnology. Plasmid pEpoE-luc containing a HIF-1 binding site (5′-T**ACGTG**CT-3′) and pEpoEm-luc with mutated site (5′-T**AAAAG**CT-3′) was generously provided by Dr. Franklin Bunn (Hematology-Oncology Division, Brigham & Women's Hospital, Harvard Medical School, Boston, MA). SB203580, PD98059 and LY294002 were purchased from TOCRIS.

2.2. Cell culture and treatment

DU145, a human prostate carcinoma, was maintained in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37 °C with 95% air and 5% CO_2. For treatment of cells with sodium arsenite, cobalt chloride and sodium orthovanadate, cells were seeded in culture dishes in medium and grown overnight. Thereafter, these metals were added to the culture medium, and cells were incubated further for the time periods as indicated in each experiment. Pretreatment of cells with compound C, LY294002, SB203580, PD98059, GSH, catalase, and NAC were performed for 1 h before the addition of each metal.

2.3. Adenovirus-mediated gene transfer

C-MYC-tagged AMPK wild type α subunit (WT) and a dominant negative form (DN), in which Asp^{157} was replaced with alanine, were generated by polymerase chain reaction as previously described [27]. Recombinant adenovirus was prepared and purified as previously described [19]. Infections with Ad-αWT or Ad-αDN were conducted in phosphate-buffered saline for 30 min at 37 °C, and then fresh medium was added.

2.4. VEGF ELISA assay

After exposure to sodium arsenite, vanadate and cobalt chloride for the indicated time period, the medium was removed and stored at −80 °C until assayed. VEGF concentrations were determined using ELISA kit (R&D systems), following manufacturer's instructions. Samples from three different experiments were analyzed in duplicate.

2.5. RNA Isolation and RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen). cDNA was prepared by incubating 1 μg of total RNA with 250 units of avian myeloblastosis virus-reverse transcriptase (Promega), 1 μM each dNTP, and random primer (0.05 μM) for 60 min at 37 °C. The cDNA fragment was amplified by PCR using following specific primers: VEGF, sense 5′-AGGAGGGCAGAATCATCACG-3′ and antisense 5′-CAAGGCCCACAGGGATTTTCT-3′; β-actin, sense 5′-GTGGGGCGCCCCAGGCACCA-3′ and antisense 5′-CTCCTTAATGTCACGCACCATTTC-3′; PCR was initiated in a thermal cycle programmed at 95 °C for 5 min, 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min and amplified for 25 cycles. The amplified products were visualized on 1% agarose gels.

2.6. ATP analysis

Intracellular ATP was extracted from cells and measured by the luciferin/luciferase method by using an ATP Determination Kit (Molecular Probes). The assay buffer (100 μL) containing 0.5 mM luciferin, 1.25 μg/mL luciferase, 25 mM Tris, pH 7.8, 5 mM $MgSO_4$, 100 μM EDTA, and 1 mM DTT was mixed with cell lysate (20 μL). Luminescence was analyzed and normalized by using cellular proteins.

2.7. Measurement of ROS

Cells were incubated with 10 μM of 2′,7′-dichlorofluorescein diacetate (Sigma) for 30 min, harvested by trypsinization, collected by centrifugation, and resuspended in PBS containing 2 μg/mL propidium iodide (Sigma). After sorting out the viable cells, fluorescence intensity was measured by flow cytometry (Becton–Dickinson) using excitation and emission wavelengths of 488 and 525 nm, respectively.

2.8. Assessment of cell viability

Cell viability elicited by exposure of metals was evaluated by measuring MTT reduction as previously described [28].

3. Results

3.1. Arsenite, vanadate, and cobalt activate AMPK in cancer cell

We first conducted an evaluation of the effects of arsenite, vanadate, and cobalt on AMPK activity in DU145 human prostate carcinoma cells. DU145 cells were exposed to 100 μM of each metal for the indicated time periods (Fig. 1A). These metals were shown to increase the level of Thr^{172} phosphorylation in the active site of the AMPK α catalytic subunit, which is crucial for enzyme activity [17]. This increase in Thr^{172} phosphorylation occurred simultaneously with an increase in the levels of phosphorylation at serine 79 of acetyl-CoA carboxylase (ACC), which is the site at which AMPK phosphorylation has been characterized most thoroughly in past studies [29]. The total amounts of the AMPK α catalytic subunit and ACC in the cells were essentially identical. Thus, our data indicates that AMPK is activated sensitively by exposure to carcinogenic metals. Arsenite and vanadate-induced AMPK activity persisted for at least 8 h, whereas cobalt-induced activation reached peak levels at the 2 h mark, and then decreased gradually. We subsequently attempted to evaluate the sensitivity of AMPK activation to different metal concentrations, and the maximum levels of AMPK activation were observed at concentrations of 50–100 μM (Fig. 1B).

3.2. Effects of the carcinogenic metals on the levels of intracellular ATP and reactive oxygen species

We subsequently investigated the mechanisms for the metal-induced AMPK activation. AMPK is well-established to be activated sensitively under ATP-depletion conditions [17], and thus we initially measured the levels of intracellular ATP in

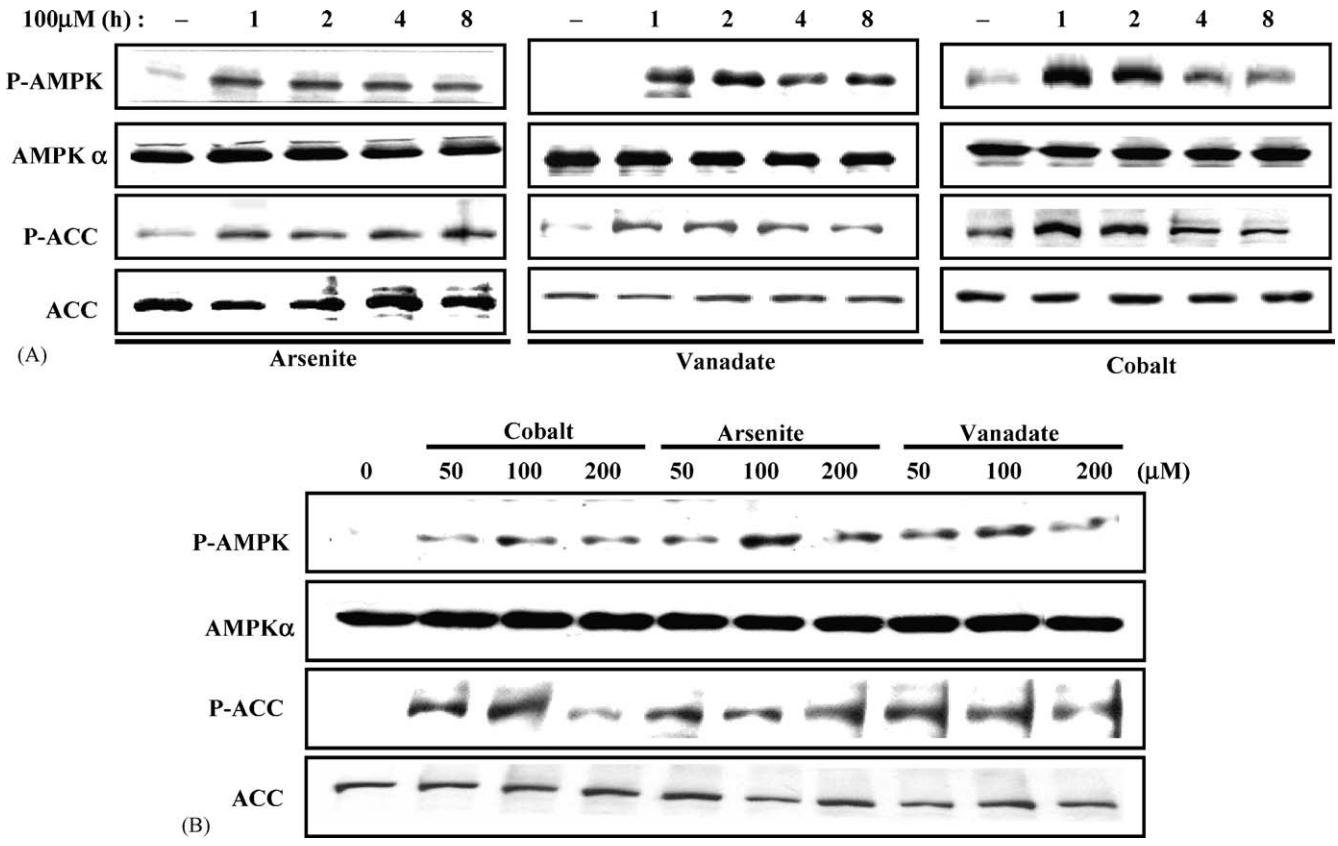

Fig. 1 – AMPK is activated by exposure to carcinogenic metals in DU145 cells. DU145 cells were exposed to arsenite, vanadate, or cobalt (100 μM) for the indicated time periods (A) or were exposed to differing concentrations of these metals for 1 h (B). Cell extracts were prepared under identical conditions, and subjected to Immunoblot assays using anti-phospho-specific ACC-Ser^{79} (P-ACC), anti-ACC (ACC), anti-phospho-specific AMPKα-Thr^{172} (P-AMPK), and anti-AMPKα (AMPKα) antibodies.

cells which had been exposed to differing concentrations of each of the tested metals for 8 h (Fig. 2A). Eight hours of cobalt treatment resulted in substantial reduction in the levels of cellular ATP, in a dose-dependent manner, with a 50% reduction occurring as the result of exposure to a 25–100 μM concentration of cobalt. We also discovered that the effects of cobalt (100 μM) occurred quite rapidly, reducing cellular ATP levels within 1 h of exposure (data not shown). By way of contrast, both arsenite and vanadate had relatively little effect on ATP levels, inducing reductions of only ~10% (Fig. 2A). This suggests that the depletion of ATP is responsible for the cobalt-induced activation of AMPK, whereas treatment with arsenite and vanadate activates AMPK by some mechanism other than ATP depletion. We further suspected that ROS might trigger an upstream signal which leads to the activation of AMPK in the arsenite- and vanadate-treated cells, due to the following reasons. Firstly, carcinogenic metals such as arsenite and vanadate are known to generate ROS, which perform a critical function in the context of metal-induced carcinogenesis [4,23–26,30]. Secondly, hydrogen peroxide is capable of inducing VEGF expression [31,32]. Thirdly, we have also demonstrated, in previous studies, that AMPK is activated sensitively by exposure to hydrogen peroxide [33]. Thus, we next attempted to assess intracellular ROS levels in the DU145 cells after exposure to each of the tested metals, using 2′,7′-dichloro-fluorescein-diacetate (DCFH-DA). Our results indicated that vanadate and arsenite, but not cobalt, generated substantial quantities of ROS, after 1 h of exposure to the indicated concentrations of each of these metals (Fig. 2B). However, when cobalt treatment was applied, the intensity of the DCF fluorescence also increased, at concentrations greater than 250 μM (data not shown).

In order to determine whether ROS plays a direct part in the AMPK signaling pathway, we subsequently assessed the effects of antioxidants including catalase, reduced glutathione (GSH), or *N*-acetyl-cysteine (NAC). Our results indicated that pretreatment with antioxidants almost completely inhibited the arsenite- and vanadate-induced activation of AMPK, but had no effects on cobalt-induced AMPK activation (Fig. 2C). Taken together, these data suggest that vanadate and arsenite activate AMPK via ROS generation without significantly altering the levels of cellular ATP. In the case of cobalt treatment, the depletion of ATP appears to constitute a primary factor in the activation of AMPK.

3.3. *AMPK activity is crucial for metal-induced VEGF expression*

We next assessed the expression levels of VEGF, a key regulator of both tumor progression and angiogenesis, in the culture media of DU145 cells which had been exposed for 12 h to either arsenite, vanadate, or cobalt. The amount of

secreted VEGF, as determined with a commercial ELISA kit, was found to have increased by ~2–4-folds under the condition, and these induction events were abrogated when the endogenous AMPK activity was inhibited as the result of the adenovirous-mediated expression of AMPK in its dominant negative form (Ad-αDN) (Fig. 3A). In conjunction with the amount of secreted VEGF protein, the levels of VEGF transcripts also increased in response to exposure to the metals, as evidenced by the results of quantitative RT-PCR (Fig. 3B). Also, the inhibition of AMPK effected significant reductions in the levels of VEGF mRNA (Fig. 3B).

Considering the role of cellular ATP or ROS levels in the activation of AMPK in the metal-treated cells, we then attempted to determine whether the AMPK activation signal could be associated directly with VEGF expression. As was described in Fig. 2C, we exposed DU145 cells to each of the tested metals for 12 h, in either the presence or absence of catalase, GSH, or NAC, and then measured the VEGF mRNA level (Fig. 3C). The antioxidants were determined to have efficiently blocked vanadate-induced VEGF mRNA expression. In the case of arsenite treatment, GSH and NAC blocked VEGF expression more potently than did catalase. In a sharply contrasting result, the antioxidants exerted no significant effects on VEGF mRNA expression when cells were induced by cobalt, under our experimental conditions (Fig. 3C). Our data thus far, then would appear to suggest that vanadate clearly relies on a ROS-sensitive mechanism to induce AMPK and VEGF expression. In the case of arsenite treatment, the role of ROS has proven relatively elusive, as catalase was not determined to attenuate the expression of VEGF as effectively as did the other antioxidants, GSH and NAC. Therefore, intracellular GSH levels may actually be more critical than ROS itself in the case of arsenite-induced VEGF expression. This possibility is discussed further in Section 4. In the case of cobalt treatment, it appears likely that AMPK and VEGF is induced via a ROS-independent mechanism under our experimental conditions. Overall, these results suggest that the activity of AMPK is a prerequisite for the carcinogenic metal-induced expression of VEGF mRNA.

3.4. AMPK activity is crucial for HIF-1 activity induced by cobalt and vanadate

In an attempt to gain further insight into the role of AMPK in the context of metal-induced VEGF expression, we then attempted to assess the effects of metal exposure on HIF-1, a major VEGF transcription factor. The metals were determined to strongly induce the expression of HIF-1α, albeit with different kinetics; protein induction as the result of cobalt exposure was observed within 1 h, whereas HIF-1α was

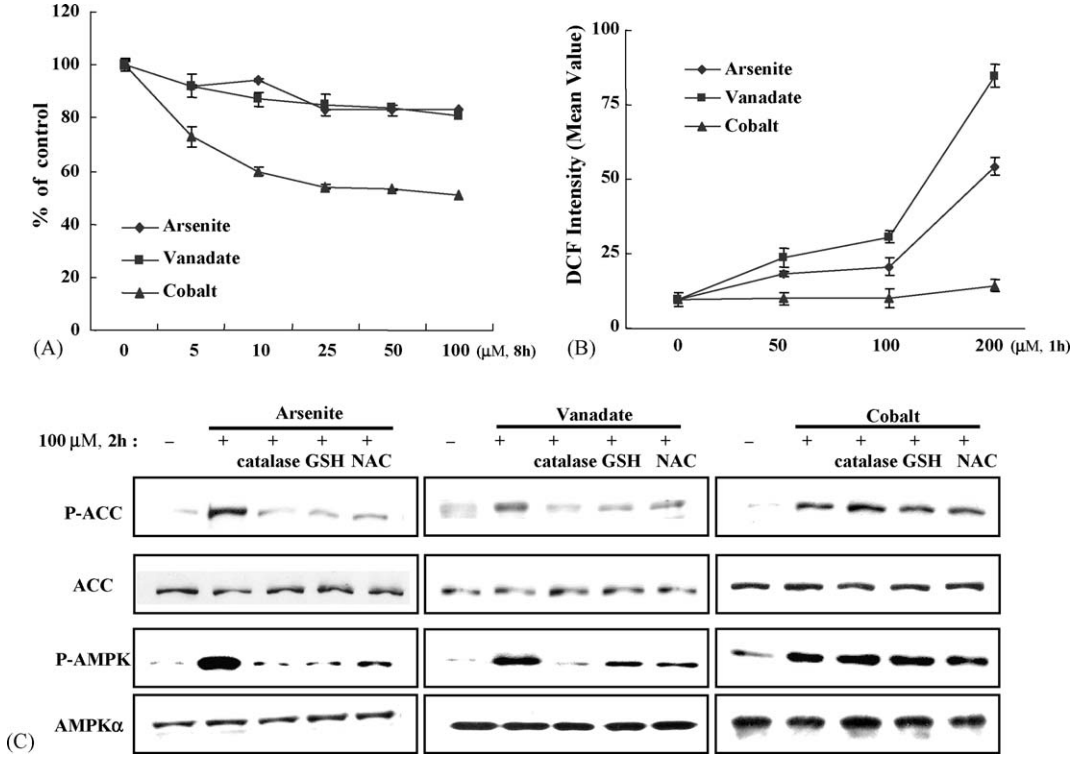

Fig. 2 – Effects of the metals on cellular ATP and ROS levels. (A) DU145 cells were treated with different concentrations of arsenite, vanadate or cobalt for 8 h, and the cellular ATP concentrations were determined as was described in Section 2. The results are expressed as the means ± S.E. from at least three experiments. (B) After exposure to different arsenite, vanadate, or cobalt concentration for 60 min, the cells were incubated for 30 min with a 10 μM concentration of DCFH-DA, and changes in the fluorescence intensity were evaluated via fluorescence-activated cell scanning analysis. The data represent the means ± S.E. for 3 determinants (C). After 30 min of pretreatment with 1000 units/mL of catalase, 10 mM of reduced glutathione (GSH), or 10 mM of N-acetyl-cystein (NAC), the cells were exposed to arsenite, vanadate, or cobalt for 2 h. Then, the phosphorylation levels of ACC (P-ACC) and AMPK (P-AMPK) were assessed via Immunoblot assays.

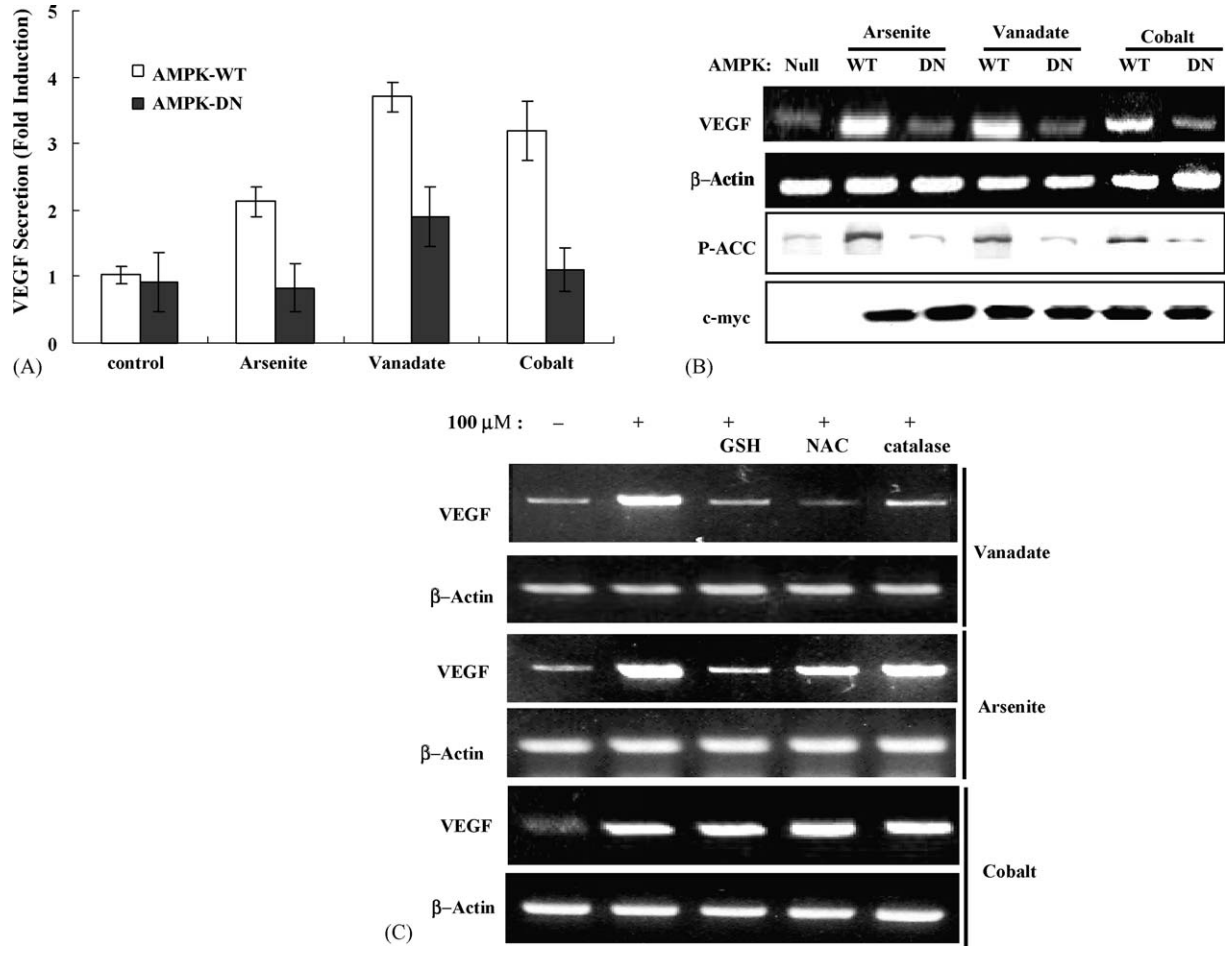

Fig. 3 – AMPK activity is necessary for the metal-induced VEGF expression. (A) DU145 cells were infected with c-MYC-tagged AMPK wild type (Ad-αWT) or dominant negative form of AMPK (Ad-αDN) at a concentration of 100 plaque-forming units/cell for 24 h, and then further exposed to 100 μM of arsenite, vanadate, or cobalt for 12 h. The levels of secreted VEGF protein in the culture media were measured with a commercial VEGF ELISA assay kit. The data represent the means ± S.E. for 6 determinants. (B) Under the identical condition, we conducted RT-PCR in order to determine the mRNA levels of VEGF and β-actin genes. Null, adenovirus with no exogenous gene; WT, adenovirus with AMPK α wild type; DN, adenovirus with dominant negative α subunit of AMPK. (C) After 30 min of pretreatment with 1000 units/mL of catalase, 10 mM of reduced glutathione (GSH), or 10 mM of *N*-acetyl-cystein (NAC), the cells were exposed to arsenite, vanadate, or cobalt for 12 h. Total RNA was then extracted from these cells, reverse-transcribed, and subjected to RT-PCR, using the primers for VEGF and β-actin genes.

detected only after 4–8 h of exposure to either arsenite or vanadate (Fig. 4A, left panel). The maximum effects of these metals on the expression of HIF-1α were observed at 50–100 μM (Fig. 4A, right panel).

In order to determine whether AMPK modulates the transcription of VEGF in an HIF-1-dependent manner, we have attempted to examine the effects of the inhibition of AMPK on HIF-1α protein levels, as the functional activity of HIF-1 is known to be regulated principally by the accumulation of HIF-1α. The inhibition of endogenous AMPK activity by infection with Ad-αDN has been shown to lead to the specific suppression of arsenite-induced and vanadate-induced expression of HIF-1α. However, AMPK inhibition was found to have no effect on cobalt-induced HIF-1α expression (Fig. 4B).

To further characterize the functional activity of HIF-1 in response to carcinogenic metal exposure, the cells were transiently transfected with a pEpoE-luc luciferase reporter gene construct, which contained an HIF-1-binding element (5′-TACGTGCT-3′), and were exposed to each of the metals for 12 h. We used pEpoEm-luc with mutated sites (5′-TAAAAGCT-3′) as a negative control. As shown in Fig. 4C, the cobalt and vanadate significantly induced the HIF-1 transcriptional activity by up to ~5–7-fold, and the induced HIF-1 activities were diminished substantially by AMPK-DN or by a specific AMPK inhibitor compound C [34]. By way of contrast, arsenite treatment was not determined to induce luciferase activity to any detectable degree. These results indicate that the induction of HIF-1α protein due to arsenite exposure did not result in the formation of functional HIF-1. As a result,

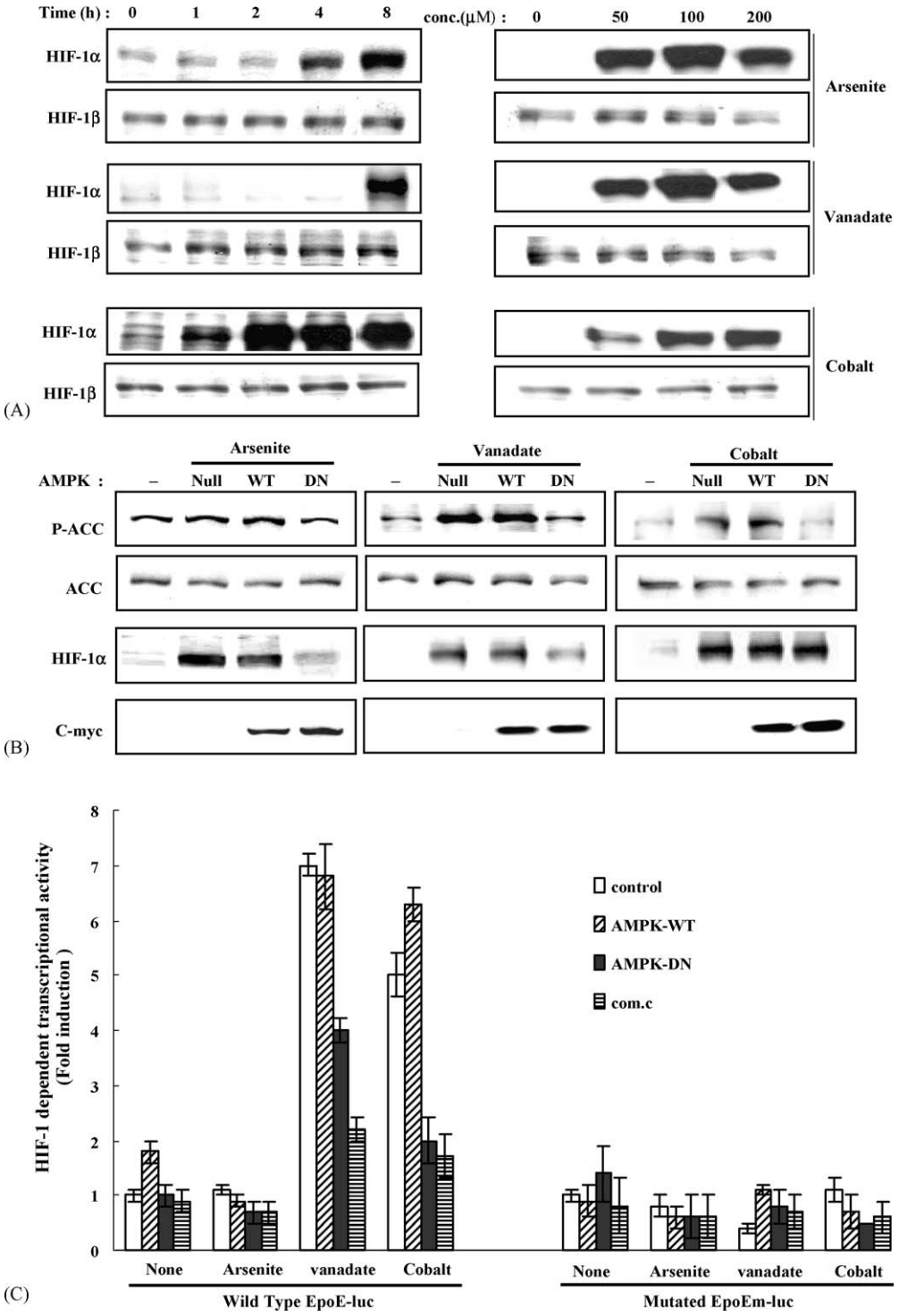

Fig. 4 – The role of AMPK on the metal-induced HIF-1α expression and transcriptional activity. (A) DU145 cells were exposed to arsenite, vanadate, or cobalt (100 μM) for the indicated time periods (left panel) or were exposed to differing concentrations of metals for 8 h (right panel). Protein extracts were prepared and subjected to Immunoblot assays employing anti-HIF-1α antibodies. (B) DU145 cells were infected with Ad-αWT or Ad-αDN at a concentration of 100 plaque-forming units/cell, then incubated for an additional 24 h. These cells were then exposed to arsenite, vanadate, or cobalt for 8 h, and the levels of HIF-1α, HIF-1β, phosphorylation of ACC (P-ACC), and c-myc were determined via Immunoblot assays. (C) DU145 cells were co-transfected with pEpoE-luciferase reporter constructs, or mutated pEpoEm-luc and pcDNA3 containing AMPKα wild type (WT) or the dominant negative α subunit (DN). Twenty-four hours after transfection, the cells were exposed for 8 h to 100 μM of arsenite, vanadate, or cobalt, in the absence or presence of specific AMPK inhibitor compound C, after which we measured luciferase activity. The data represent the means ± S.E. for 6 determinants. Com.c; compound C.

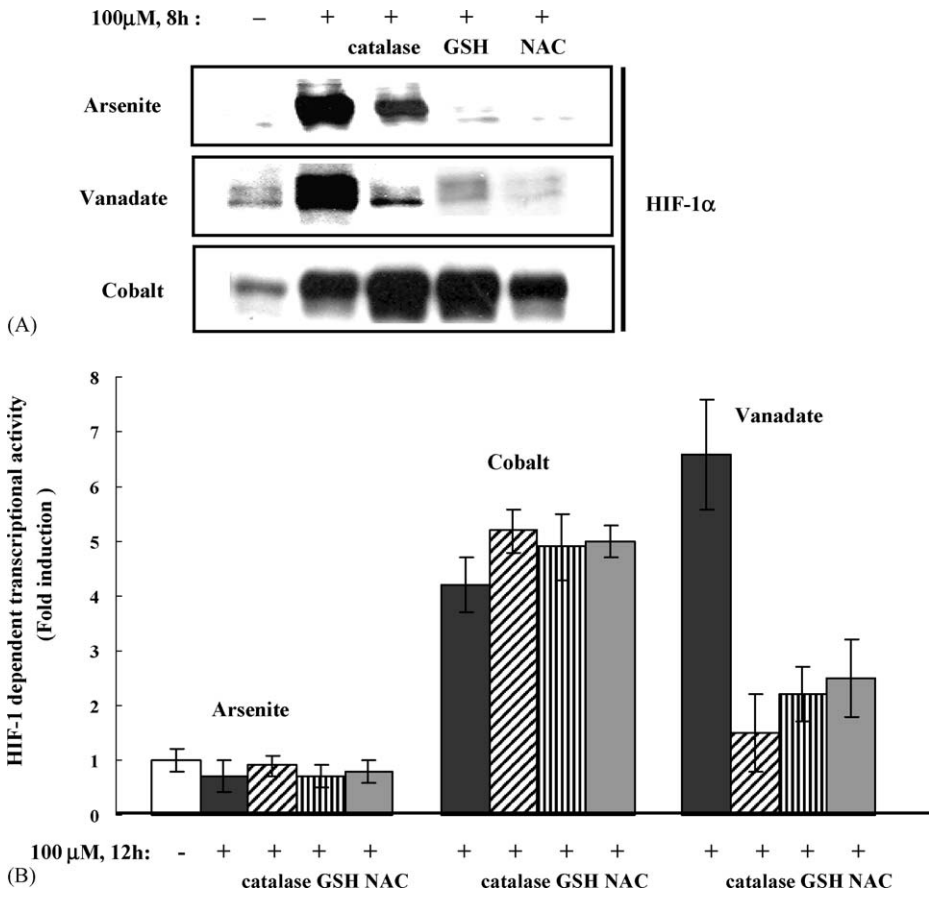

Fig. 5 – Effects of anti-oxidants on the metal-induced HIF-1α expression and HIF-1 activity in the DU145 cells. (A) After 30 min of pretreatment with 1000 units/mL of catalase, 10 mM of reduced glutathione (GSH), or 10 mM of *N*-acetyl-cystein (NAC), the cells were exposed to arsenite, vanadate, or cobalt for 8 h. Then, HIF-1α protein levels were assessed via Immunoblot assays. (B) The cells were transiently transfected with the pEpoE-luc reporter construct. After 24 h of transfection, the cells were exposed to 100 μM of arsenite, vanadate, or cobalt for 12 h, in the absence or presence of catalase (1000 units/mL), GSH (10 mM), or NAC (10 mM). The cell lysates were then subjected to luciferase activity assays. The data are presented as means ± S.E. from 6 determinations.

arsenite-induced VEGF expression did not appear to require a functional HIF-1, but AMPK activity was still clearly necessary for the process. Taken together, our data thus far suggest that AMPK is able to regulate the expression of VEGF mRNA in a HIF-1-dependent or -independent manner, depending on the stimuli applied.

We subsequently tested the role of ROS in HIF-1 regulation in the metal-treated cells. DU145 cells were exposed to each metal in either the presence or absence of antioxidants, and then we measured the protein level of HIF-1α (Fig. 5A) and the transcriptional activity of HIF-1 (Fig. 5B). The antioxidants were determined to have efficiently blocked vanadate-induced HIF-1α expression and HIF-1 transcriptional activity, thereby highlighting the significant role played by ROS in the AMPK→HIF-1→VEGF signal axis. In contrast, antioxidants exerted no effects on these parameters when cells were induced by cobalt. In the case of arsenite treatment, GSH and NAC blocked HIF-1α expression more potently than did catalase (Fig. 5A). However, again the functional activity of HIF-1 was not associated with the accumulation of the HIF-1α protein (Fig. 5B).

3.5. *The carcinogenic metal-induced AMPK signaling pathway is independent of the p38 MAPK and PI-3 kinase pathways*

We then attempted to elucidate the AMPK signaling pathway by characterizing the cross-talk occurring between signaling pathways, which has also been implicated in the metal-induced expression of HIF-1α or VEGF. Carcinogenic metals influence a variety of signaling molecules, including PI-3 kinase/AKT, ERK, p38, or JNK [4,23–26,28,35]. Among these, p38 MAPK has most recently been implicated in the arsenite-induced expression of VEGF mRNA [26], whereas PI-3 kinase/AKT has been reported to be crucial in the HIF-1α expression induced by vanadate and cobalt exposure [22,23]. However, the actual roles played by these kinases appear to be both highly cell-type specific and stimuli-dependent. For this reason, we initially examined the activation kinetics of these kinases, as well as their functional roles in the DU145 cells. As is shown in Fig. 6A, arsenite was found to rapidly activate p38 MAPK in the DU145 cells, as assessed by the levels of p38 MAPK-Thr^{180}/Tyr^{182} phosphorylation. Vanadate and cobalt rapidly induced

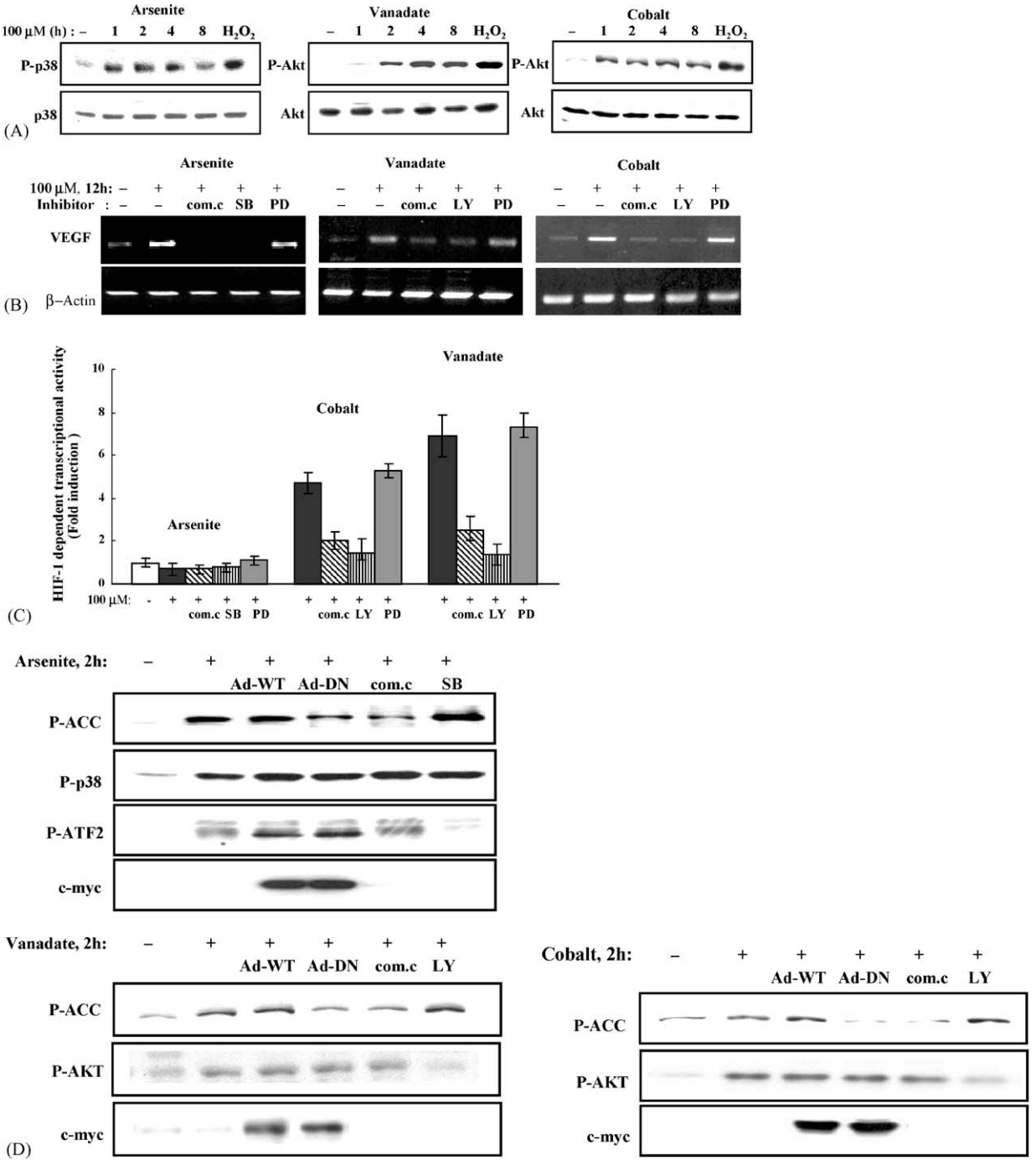

Fig. 6 – The AMPK signal pathway is independent of p38 MAP kinase or PI-3 kinase in the metal-exposed DU145 cells. (A) DU145 cells were exposed to arsenite, vanadate, or cobalt for the indicated times, and then the phosphorylation levels of p38-Thr^{180}/Tyr^{182} (P-p38), AKT-Ser^{473} (P-AKT), as well as the total amounts of p38 and AKT were determined via immunoblot analysis. (B) The cells were exposed to the indicated metals, in the presence of 20 μM of compound C, SB203580, or LY294002, for 12 h. Under each of the experimental conditions, the mRNA levels of VEGF and β-actin were determined via RT-PCR. (C) After transfection using pEpoE-luciferase reporter constructs, the cells were treated with each of the metals in the presence of kinase inhibitors, and we measured luciferase activity. The data is expressed as the means ± S.E. from 6 determinations. (D) The DU145 cells were infected with c-MYC-tagged Ad-αWT or Ad-αDN at a concentration of 100 plaque-forming units/cell for 24 h, then exposed to 100 μM of arsenite, vanadate, or cobalt, in both the absence and presence of Compound C (20 μM), SB203580 (20 μM), or LY294002 (20 μM) for 2 h. Then, the phosphorylation levels of ACC-Ser^{79} (P-ACC), p38-Thr^{180}/Tyr^{182} (P-p38), ATF-2-Thr$^{69/71}$ (P-ATF2), and AKT-Ser^{473} (P-AKT) were assessed via Immunoblot assay. Com.c, compound C; SB, SB203580; LY, LY294002.

heightened AKT-Ser^{473} phosphorylation levels (Fig. 6A). Subsequently, in order to determine the functional roles played by PI-3 kinase and p38 MAPK, the DU145 cells were pre-treated with inhibitors for each kinase (LY294002 for PI-3 kinase and SB203580 for p38 MAPK) prior to their exposure to each of the tested metals. The inhibition of PI3-kinase due to LY294002 pretreatment was found to have blocked the increase in the levels of VEGF mRNA, as well as the HIF-1 transcriptional activity which was induced by treatment with cobalt or vanadate (Fig. 6B and C). The arsenite-induced VEGF mRNA levels were decreased markedly as the result of SB203580 pretreatment (Fig. 6B). As a negative control, we also assessed the effects of PD98059, an ERK inhibitor, and we determined there to be no specific inhibition of carcinogenic metal-induced VEGF expression or of HIF-1 transcriptional activity (Fig. 6B and C). Overall, our data appear to indicate that PI-3 kinase is a prerequisite for the vanadate- or cobalt-induced expression of HIF-1 and VEGF, whereas p38 MAPK kinase seems to play an important role in the arsenite-induced expression of VEGF in the DU145 cells.

We next attempted to determine whether the AMPK signaling pathway could be correlated with p38 MAPK or PI3-kinase/AKT in the DU145 cells which were exposed to the carcinogenic metals (Fig. 6D). In order to characterize the relations between AMPK, PI-3 kinase and p38 kinase, the DU145 cells were pretreated with compound C, LY294002, SB203580, or infected with Ad-αWT or Ad-αDN, then exposed to each of the tested metal for a period of 2 h. The inhibition of AMPK by either compound C or Ad-αDN had no effects on the vanadate- and cobalt-induced levels of AKT-Ser^{473} phosphorylation. Conversely, LY294002 was determined not to alter the metal-induced levels of ACC-Ser^{79} phosphorylation, thereby suggesting that these two pathways are independent of each other. Similarly, the inhibition of AMPK had no detectable effects on the activity of p38 MAPK as induced by arsenite, as was evidenced by the phosphorylation levels of p38 and its substrate, ATF2. Furthermore, SB203580 was also determined not to alter the level of ACC-Ser^{79} phosphorylation. Thus, our results indicate that the AMPK, PI-3 kinase, and p38 MAPK signal pathways are required for the HIF-1α and VEGF expression induced by each of the carcinogenic metals tested, but that each of these pathways transmits unique signals, each in an independent fashion.

3.6. *Effects of AMPK inhibition on cell viability in DU145 cells exposed to carcinogenic metals*

The metals tested in the present study shows the carcinogenic potentials, in part due to their ability to induce VEGF expression, but paradoxically these metals also exert cytotoxic effects in various cells. To this end, we finally examined the role of AMPK in the context of cell viability (Fig. 7). After infection with Ad-αWT or Ad-αDN for 24 h, DU145 cells then were exposed to 50 and 100 μM concentrations of arsenite, vanadate, and cobalt for 24 h, and we then conducted MTT assays. The metals exhibited differential degrees of cell toxicity, and arsenite was determined to be the most toxic among them, resulting in a 20–30% reduction in cell viability. In all of the tested metals, AMPK inhibition resulted in a dramatic reduction in cell viability, highlighting the critical role played by AMPK with regard to the protection of the cell against toxic metals.

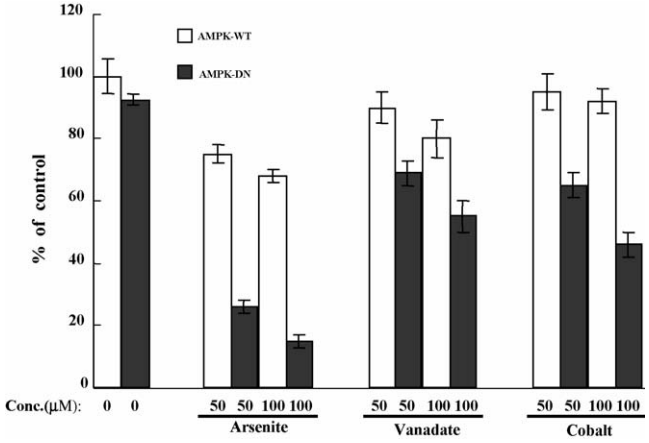

Fig. 7 – Effects of AMPK inhibition on cell viability in metal-exposed DU145 cells. After infection with AMPKα wild type (WT) or the dominant negative α subunit (DN) for 24 h, cells were exposed to 50 and 100 μM concentrations of arsenite, vanadate, or cobalt for 24 h, and we then determined cell viability via MTT reduction. The results are expressed as the means ± S.E. from 6 experiments.

4. Discussion

The risk of cancer in a given individual is determined by both genetic and environmental interactions. Most cancers are associated with an occupational, viral, nutritional, or iatrogenic etiology. Inevitably, metals are a concern of the highest priority with regard to human exposure, by virtue of their extensive distribution and wide usage in modern society [36]. The International Agency for Research on Cancer has assessed and reported the carcinogenic potential of a host of metals, and has confirmed that arsenite is a carcinogen in humans [37]. Animal studies, as well as epidemiological studies, have also shown the carcinogenic potential harbored by both cobalt and vanadate [4,37]. In the present study, we have attempted to characterize the mechanisms inherent to the expression of VEGF by the aforementioned metals in cancer cells and, furthermore, have demonstrated the critical role played by AMPK in this process. The concentrations of metals used in this study are relatively high as compared to the level generally found in the environments [53]. However, depending on the tested cell lines and conditions, the applied dose of metals varies in the wide range of 0.1 to ≥300 μM. In fact, similar or even higher dose of metals has been used to characterize a number of signal pathways including mitogen-activated protein kinase and transcription factors, and such results were reported to be relevant to epidemiologic studies [53,55].

We have summarized our results and the proposed role of AMPK in a diagram (Fig. 8). Here, we have shown that the induction of VEGF by cobalt and vanadate is mediated in an HIF-1-dependent manner, although the mechanisms underlying these effects differ. However, the induction of VEGF by arsenite occurred in an HIF-1-independent manner. It has

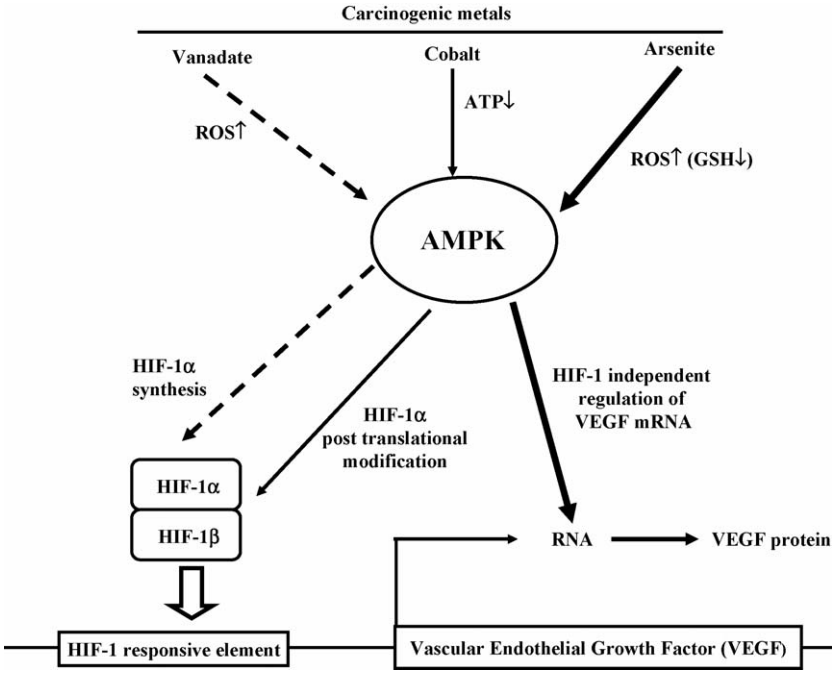

Fig. 8 – Proposed scheme for AMPK activation leading to VEGF induction. Our results are summarized in a diagram. Vanadate (a dotted line), Cobalt (a solid line), and Arsenite (a bold line) activate AMPK via multiple mechanisms, which increase the expression of VEGF in either an HIF-1-dependent or -independent manner.

been previously shown that carcinogenic metals increase HIF-1α in various cancer cells [38], and we also observed the effects of the tested metals in other cells than DU145 (data not shown). Cobalt is well-known to stabilize HIF-1α protein under normoxic conditions [39]. By way of contrast, it appears quite likely that vanadate exerts a stimulatory effect on the synthesis of the HIF-1α protein, as evidenced by the following observations. Firstly, the kinetics of vanadate-induced HIF-la expression are quite different from the kinetics of cobalt-induced expression (Fig. 4A); HIF-1α was detected within 1 h of exposure to cobalt, whereas it required at least 8 h to detect protein expression after vanadate treatment, thereby indicating that the mechanisms of each process were different. Secondly, we recently demonstrated that the vanadate-induced expression of HIF-1α was cycloheximide-sensitive [25]. Thirdly, a recent report has shown that the PI-3 kinase/Akt/mTOR pathway was a prerequisite for the expression of HIF-1α as the result of vanadate treatment [23]. This signal pathway was originally implicated in the stimulation of HIF-1α protein synthesis by hormones, under normoxic conditions, thereby overwhelming the oxygen-dependent degradation of the HIF-1α protein [13,40]. In a manner similar to that associated with vanadate, it was reported that arsenite induced the expression of HIF-1α in a PI-3 kinase/Akt-dependent manner [41], but our results showed that arsenite-induced HIF-1 exhibited no functional transcription activity (Figs. 4C, 5B and 6C). This observation is fairly analogous to that of the effects of proteasome inhibitor treatment on the expression of HIF-1α; although the proteasome inhibitor was shown to stabilize the HIF-1α protein under normoxic conditions, the functional HIF-1 transcriptional activity was not detected [42], and also arsenite induce HIF-1α expression in various cells, but there were no specific transcriptional activities in those cells [26,38,43]. Therefore, it seems reasonable to assume that arsenite did not stimulate the signal pathway which results in whatever auxiliary modifications are required for full HIF-1 activity. At this point, the precise mechanism by which VEGF expression is induced by arsenite remains somewhat elusive. Overall, then, our data indicate that the mechanisms underlying the expression of VEGF by cobalt, vanadate, and arsenite all differ (Fig. 8). As the expression levels of VEGF by the aforementioned metals were abrogated substantially due to the inhibition of AMPK, it appears rather likely that AMPK can regulate VEGF expression at multiple regulatory steps, possibly including HIF-1α post-translational modification, protein synthesis, and HIF-1-independent VEGF expression (Fig. 8). Therefore, the results of this study imply that AMPK may constitute a critical regulator of VEGF expression under various conditions, and AMPK may also contribute to the development of cancer via the regulation of critical features of cancer cell adaptation, including tumor angiogenesis.

The effects of ROS are relevant to carcinogenesis, but recent data also indicates that metal-induced alterations in the signal transduction pathway also perform a function in the etiology of cancer either independently of, or in concert with, ROS [4,31,45]. In fact, some investigators have reported that cobalt is capable of inducing oxidative stresses [22,46], whereas others have indicated that cobalt could increase the expression of hypoxia-regulated genes in A549 cells, in a ROS-independent fashion [47]. Under our experimental conditions, the generation of ROS by cobalt (100 μM) was not as distinctive as that induced by arsenite or vanadate, with regard to DCF oxidation (Fig. 2B). Moreover, anti-oxidants were not determined to block the activation of AMPK (Fig. 2C), the

activity of HIF-1 (Fig. 5B), or the expression of VEGF mRNA under cobalt-treatment conditions (Fig. 3C). This suggests that factors other than ROS, probably ATP depletion, appear to be more critical under such conditions, as other reports have indicated that chronic cobalt exposure effects a reduction in the rate of mitochondrial ATP production in rat myocardial tissue [48].

In the case of vanadate treatment, ROS generation was determined to be rather prominent, and its role with regard to AMPK activation was quite clear (Fig. 2). Arsenite was also determined to generate ROS in a quite distinctive manner (Fig. 2B), but an additional mechanism also appears to be involved in the arsenite-induced expression of VEGF. It has been reported that arsenite generates oxidative stress via the attenuation of intracellular GSH levels. Arsenite has also been shown to bind to free thiol groups on critical cellular proteins, therefore modifying their functions [49,50]. As is shown in Figs. 3C and 5A, GSH and NAC both were determined to block arsenite-induced VEGF mRNA and HIF-1α expression more efficiently than did the hydrogen peroxide scavenger, catalase. Therefore, it remains possible that arsenite may exert its effects by virtue of a fairly complicated mechanism, which involves the modulation of intracellular GSH levels, in addition to the generation of ROS.

The metals employed in the present study had been reported to exert profound effects on cancer development. However, they also, paradoxically, exert apoptotic effects in a variety of cells [51,52]. It remains a matter of some controversy, however, as to how these opposite effects are regulated in cases of metal exposure. In addition to its contribution to the angiogenic potential of endothelial cells, VEGF has been shown to exert both autocrine and paracrine effects in a variety of cancer cells [56]. The overproduction of VEGF with the concomitantly expressed receptors results in an enhancement of the growth and survival of cancer cells via a VEGF-dependent internal autocrine loop mechanism. In summary, our data appear to strongly suggest that AMPK protects cells against the apoptotic effects exerted by the tested metals, simultaneously mediating their carcinogenic activity, in part, via the regulation of VEGF expression.

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