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Research Paper

Arsenite-induced stress signaling: Modulation of the phosphoinositide 3′-kinase/Akt/FoxO signaling cascade ☆

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ABSTRACT

FoxO transcription factors and their regulators in the phosphoinositide 3′-kinase (PI3K)/Akt signaling pathway play an important role in the control of cellular processes involved in carcinogenesis, such as proliferation and apoptosis. We have previously demonstrated that physiologically relevant heavy metal ions, such as copper or zinc ions, can stimulate this pathway, triggering phosphorylation and nuclear export of FoxO transcription factors. The present study aims at investigating the effect of arsenite on FoxO transcription factors and the role of PI3K/Akt signaling therein. Exposure of HaCaT human keratinocytes to arsenite resulted in a distinct decrease of glutathione levels only at cytotoxic concentrations. In contrast, a strong phosphorylation of FoxO1a/FoxO3a and Akt was observed at subcytotoxic concentrations of arsenite in HaCaT human keratinocytes. A time- and concentration-dependent increase in phosphorylation of FoxO1a and FoxO3a at sites known to be phosphorylated by Akt as well as phosphorylation of Akt at Ser-473 was detected. These phosphorylations were blunted in the presence of wortmannin, pointing to the involvement of PI3K.

Introduction

FoxO (forkhead box, class O) transcription factors (with three of its four isoforms, FoxO1a, 3a and 4, present in most human cells) are stress-responsive regulators of the expression of numerous genes, thereby controlling the expression of key proteins in proliferation and cell cycle regulation, in fuel metabolism and antioxidant defense [1]. FoxO activity and subcellular localization was demonstrated to be modulated by reactive oxygen species (ROS), such as H_2O_2 [2–4], and further stressful stimuli, including metal ions [5,6]. FoxOs, in turn, regulate the expression of antioxidant enzymes such as MnSOD [3], catalase [7], or selenoprotein P [8,9], as well as proteins involved in metal homeostasis, such as ceruloplasmin [10].

Due to their role in regulation of apoptosis and cell proliferation, FoxOs are considered tumor suppressors [11]. FoxOs are regulated by posttranslational modification, most prominently phosphorylation and acetylation: phosphorylation by the phosphoinositide 3′-kinase (PI3K)-dependent Ser/Thr kinase Akt causes inactivation and nuclear exclusion of FoxOs [12].

In the present study, we set out to test for a modulation of FoxO phosphorylation and activity by arsenite, a known human carcinogen: the International Agency for Research on Cancer (IARC) has classified arsenic, as found in drinking-water, as a group 1 carcinogen, as there is "sufficient evidence of carcinogenicity" in humans that arsenic containing drinking-water causes cancers of the urinary bladder, lung and skin [13,14].

Arsenic has previously been shown to stimulate PI3K/Akt [15], suggesting that FoxO phosphorylation might indeed occur.

One consequence characteristic of oral exposure to arsenic is the development of skin lesions, including hyperkeratosis and Bowen's disease, a precursor of squamous cell carcinomas [16]. In the present study, we therefore investigated the effects of arsenite on FoxO phosphorylation using HaCaT human keratinocytes.

Materials and methods

Reagents

All chemicals were from Sigma-Aldrich (Oakville, ON, Canada), if not mentioned otherwise. Wortmannin stock solutions (0.2 mM in DMSO) were held at −20 °C; sodium arsenite was held as a stock solution of 100 mM in water at 4 °C. For use, stock solutions were diluted into Hanks' balanced salt solution (HBSS).

Cell culture and treatment of cells

HaCaT human immortalized keratinocytes [17] were a kind gift from Prof. P. Boukamp, Heidelberg, Germany. Cells were held at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 and

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cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with (final concentrations) 10% (v/v) fetal calf serum (PAA, Etobicoke, ON, Canada), and penicillin/streptomycin (100 units/ml and 0.1 mg/ml, respectively; Sigma-Aldrich).

HaCaT cells were grown to near confluence prior to treatments. Cells were then held in serum-free medium overnight prior to treatments, washed once with PBS, followed by incubation in the presence of arsenite, copper sulfate or insulin diluted into HBSS. For inhibitor experiments, cells were pre-incubated with wortmannin for 30 min in HBSS, followed by washing cells once with PBS and exposure to arsenite or copper ions in HBSS (without wortmannin). For the pre-incubation step with wortmannin, DMSO was used as vehicle control.

Cell viability

Cell viabilities after exposure to arsenite or copper sulfate were assessed by incubating and staining viable cells at 37 °C with neutral red solution (16.5 mg/l neutral red in DMEM) for 2 h. Cells were carefully washed twice with PBS, and neutral red incorporated by cells was extracted from cells with ethanol:water:acetic acid (50:49:1 v/v/v) for at least 2 h at room temperature prior to analysis of neutral red content in extracts at 405 nm (reference wavelength: 550 nm).

Determination of glutathione and glutathione disulfide

Glutathione (GSH) and glutathione disulfide (GSSG) were determined enzymatically according to [18], with minor modifications [19]. Briefly, cells on 6 well culture dishes were lysed by scraping them in 250 µl/well of ice-cold HCl (10 mM) followed by one freeze/thaw cycle, brief sonication on ice, and centrifugation at 20,000*g* for 10 min to remove cell debris. Aliquots of the supernatants were kept for protein determination in a bicinchoninic acid (BCA)-based protein assay (Pierce/Thermo Scientific, Rockford, USA). For GSH/GSSG determination, protein was precipitated from the supernatant with 5% (w/v; final concentration) 5-sulfosalicylic acid on ice. Samples were vortexed and centrifuged at 20,000*g* for 10 min. Total glutathione (GSH plus GSSG) and, after blocking thiols with 2-vinylpyridine, GSSG were determined from the supernatant using 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of NADPH and glutathione reductase [18].

Western blotting

For analysis of Akt, FoxO1a, FoxO3a, GAPDH levels or modifications, cells were lysed in 2 × Laemmli buffer [125 mM Tris/HCl, 4% (w/v) SDS, 20% glycerol, 100 mM dithiothreitol and 0.02% (w/v) bromophenol blue, pH 6.8] after treatment, followed by brief sonication. Samples were applied to SDS–polyacrylamide gels of 10% (w/v) acrylamide, followed by electrophoresis and blotting onto nitrocellulose membranes. Immunodetection was performed using the following antibodies: anti-phospho-FoxO1a/FoxO3a (T24/T32), anti-FoxO3a, anti-Akt rabbit polyclonal antibodies were from Cell Signaling Technology (New England Biolabs, Pickering, ON, Canada). Anti-phospho-Akt (S473) rabbit polyclonal antibodies from Cell Signaling Technology and from Sigma-Aldrich were used. Anti-GAPDH mouse monoclonal antibody was from Millipore (Billerica, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were from GE Healthcare (Mississauga, ON, Canada). Incubation with the primary antibodies were performed in 5% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST); incubation with the secondary antibodies were performed in 5% (w/v) non-fat dry milk in TBST.

FoxO1a DNA binding

FoxO1a DNA binding activity was assayed employing an ELISA-based FoxO-DNA binding assay (TransAM FKHR, Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, cells were harvested and nuclear protein extracted using a nuclear extraction kit (Nuclear extraction kit, Active Motif) according to the manufacturer's protocol. Nuclear extracts were applied to 96-well plates coated with oligonucleotides containing FoxO-DNA binding elements. Bound (i.e., active) FoxO was then detected using an antibody directed against FoxO1a the binding of which was assayed employing a secondary antibody conjugated with HRP.

Results

Arsenite cytotoxicity and glutathione depletion

The potential of arsenic to induce oxidative stress in exposed cells is well established. Arsenic was previously demonstrated to be capable of generating a variety of ROS intracellularly, resulting

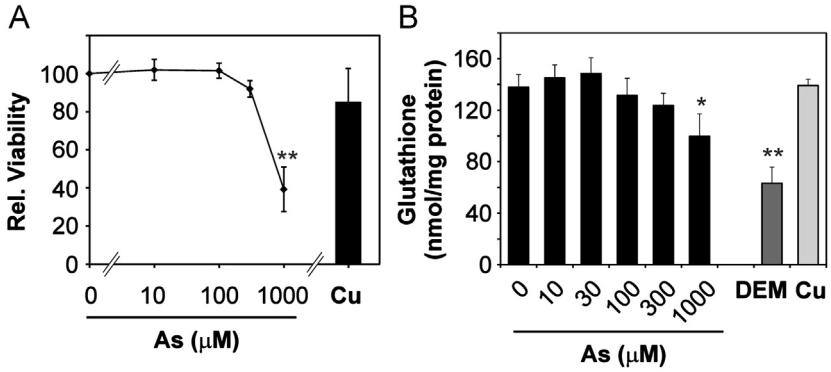

Fig. 1. *Viability and glutathione levels in cells exposed to arsenite or copper ions.* HaCaT human keratinocytes were grown to near confluence, then held in serum-free medium overnight, washed with PBS and exposed to the given concentrations of arsenite or 10 µM copper sulfate in Hanks' balanced salt solution (HBSS) for 120 min (viability) or 60 min (glutathione levels). (A) Viabilities of HaCaT cells were analyzed by neutral red staining. Cells were exposed to sodium arsenite or copper sulfate for 120 min, washed with PBS and incubated in serum-free cell culture medium for another 24 h, followed by addition of neutral red solution. Data are given as means of at least three independent experiments ± SD. (B) Glutathione levels were analyzed in cells immediately following 60 min of exposure to arsenite (10–1000 µM), copper sulfate (Cu, 10 µM) or diethyl maleate (DEM, 2 mM). Data are means of three (Cu: 2) independent experiments ± SD (Cu: range). Data significantly different from control (ANOVA with Dunnett's post-test) are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$).

in oxidative damage to DNA, lipids and proteins [20,21]. Nevertheless, only a two-hour exposure to concentrations as high as 1 mM of arsenite caused significant loss in viability of HaCaT keratinocytes 24 h post-exposure (Fig. 1A). Similarly, a significant loss in cellular glutathione (GSH) after 60 min of exposure was seen only with 1 mM arsenite (Fig. 1B). Interestingly, this loss in glutathione did not coincide with significantly increased levels of glutathione disulfide (data not shown), pointing to the induction of other mechanisms of GSH depletion, such as general glutathiolation or GSH utilization in the formation of arsenic-GSH conjugates [22,23]. While diethyl maleate, a known substrate for glutathione S-transferases, efficiently depleted glutathione, copper ions did not significantly lower glutathione concentrations (Fig. 1B).

Arsenite-induced activation of Akt: comparison to copper

Akt was significantly activated in a concentration- and time-dependent manner in HaCaT cells exposed to arsenite (Fig. 2). While Akt phosphorylation is evident after 30 min exposure to 300 μM arsenite, extensive phosphorylation was detected with 100 μM arsenite after 120 min. Interestingly, this activation is much less intense at all times than the phosphorylation induced by copper ions at lower concentrations. Akt phosphorylation was also stimulated by insulin, as expected for an insulin-responsive cell line (Fig. 2).

Arsenite-induced phosphorylation of FoxO: role of PI3K

FoxO proteins are downstream targets of Akt, phosphorylation by which inhibits FoxO transcription factor activity [12,24]. To test whether FoxOs are indeed affected by arsenite exposure, we analyzed for phosphorylation of FoxO1a and FoxO3a using an antibody recognizing phosphorylation at an Akt target site, Thr-24/Thr-32. We observed a distinct increase in the amounts of phosphorylated FoxO1a and FoxO3a after exposure to arsenite (Fig. 3). The antibody also yielded a faint signal indicating the potential phosphorylation of FoxO4 at Thr-28, the site homologous to Thr-24/Thr-32 of FoxO1a/3a. The time course of induction of phosphorylation was similar to that of Akt phosphorylation; similarly, both copper and insulin-induced phosphorylation of the FoxO proteins was much stronger than that induced by arsenite (Fig. 3).

Using wortmannin, an inhibitor of phosphoinositide 3′-kinases, we tested for a role of PI3K in arsenite-induced Akt and FoxO phosphorylation. As shown in Fig. 4A, both Akt and FoxO phosphorylation were blunted in cells pretreated with wortmannin, indicating that PI3K is required for arsenite-induced Akt and FoxO phosphorylation (Fig. 4A). In line with phosphorylation of FoxO proteins, a treatment with 100 μM arsenite slightly lowered endogenous FoxO-DNA binding activity, similar to insulin (Fig. 4B).

Discussion

Role of reactive oxygen species (ROS) in arsenite-induced Akt and FoxO phosphorylation

Regarding arsenic toxicity, the formation of ROS is currently a widely accepted and studied mechanism. ROS, such as superoxide or hydrogen peroxide, may be formed (i) during oxidation of arsenite to arsenate, (ii) result from arsenic-induced release of redox-active iron from ferritin, (iii) interactions with the respiratory chain, (iv) GSH depletion, (v) stimulation of NADPH oxidase as well as (vi) inhibition of antioxidant enzymes [13,23]. It is now widely accepted that the induction of oxidative DNA damage, diminished DNA repair, altered DNA methylation, enhanced cell proliferation and activation of stress-responsive signaling cascades contribute to arsenic-induced carcinogenesis [25]. Several signaling pathways known to regulate proliferation, such as ERK, p38 and JNK mitogen-activated protein kinases, are modulated by exposure to arsenicals (for an overview, see [26]).

However, although inducing the generation of ROS in HaCaT cells, arsenite has been demonstrated previously to be unlikely to induce activation of Akt through ROS, as antioxidants such as N-acetyl-L-cysteine or pyrrolidinedithiocarbamate as well as the NADPH oxidase inhibitor, diphenylene iodonium chloride did not inhibit arsenite-induced phosphorylation of Akt [15]. Similarly, no extensive oxidative stress was induced in our setting of HaCaT cells exposed to arsenite, as no significant decrease in GSH and increase in glutathione disulfide was detected at concentrations already causing FoxO and Akt phosphorylation (100–300 μM; Fig. 1B).

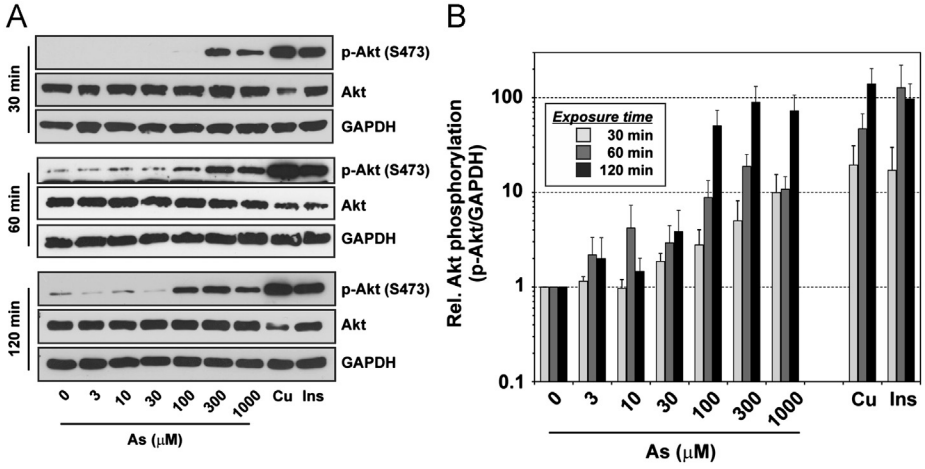

Fig. 2. *Phosphorylation of Akt in cells exposed to arsenite, copper sulfate or insulin*. HaCaT human keratinocytes were grown to near confluence, held in serum-free medium overnight, then washed with PBS and exposed to 10–1000 μM arsenite, 10 μM copper sulfate or 100 nM insulin in Hanks' balanced salt solution (HBSS) for 30, 60 or 120 min. Akt phosphorylation at Ser-473 was analyzed by Western blotting and immunodetection using a phosphospecific antibody. (A) The blots shown are representative of three independent experiments (Ins, 30 min: two) with similar results. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) staining was used to demonstrate equal loading of gels. (B) Densitometric analysis of Akt phosphorylation signals relative to GAPDH. Controls were set equal to 1. Data are means of 3 (Ins, 30 min: 2) independent experiments ± SEM. Gray, dark gray and black bars represent data for 30, 60 and 120 min exposure, respectively.

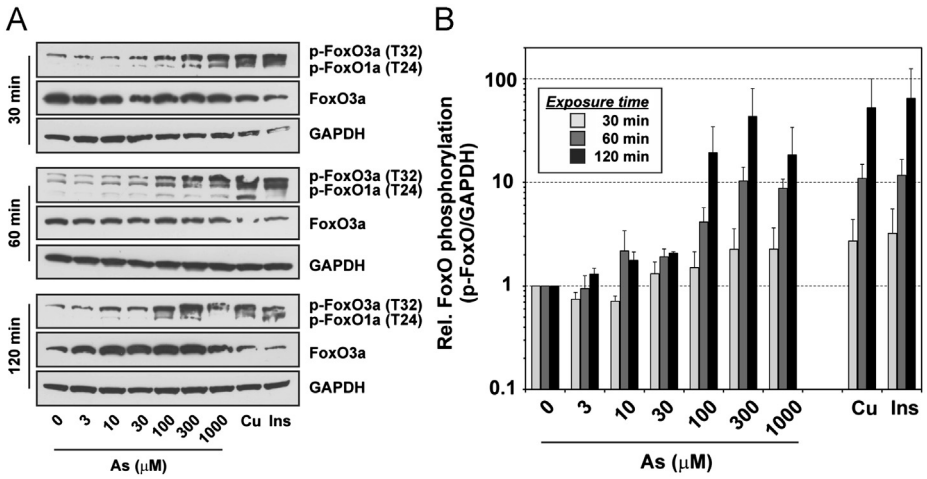

Fig. 3. *Phosphorylation of FoxO1a and FoxO3a in cells exposed to arsenite*. HaCaT human keratinocytes were grown to near confluence, held in serum-free medium overnight, then washed with PBS and exposed to 10–1000 μM arsenite, 10 μM copper sulfate or 100 nM insulin in Hanks' balanced salt solution (HBSS) for 30, 60 or 120 min. Phosphorylation of FoxO1a and FoxO3a at Thr-24 and Thr-32, respectively, was analyzed by Western blotting and immunodetection using a phosphospecific antibody. (A) The blots shown are representative of three independent experiments (Cu, 120 min: two) with similar results. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) staining was used to demonstrate equal loading of gels. (B) Densitometric analysis of FoxO phosphorylation signals relative to GAPDH. Overall FoxO phosphorylation was analyzed. Controls were set equal to 1. Data are means of 3 (Cu, 120 min: 2) independent experiments ± SEM. Gray, dark gray and black bars represent data for 30, 60 and 120 min exposure, respectively.

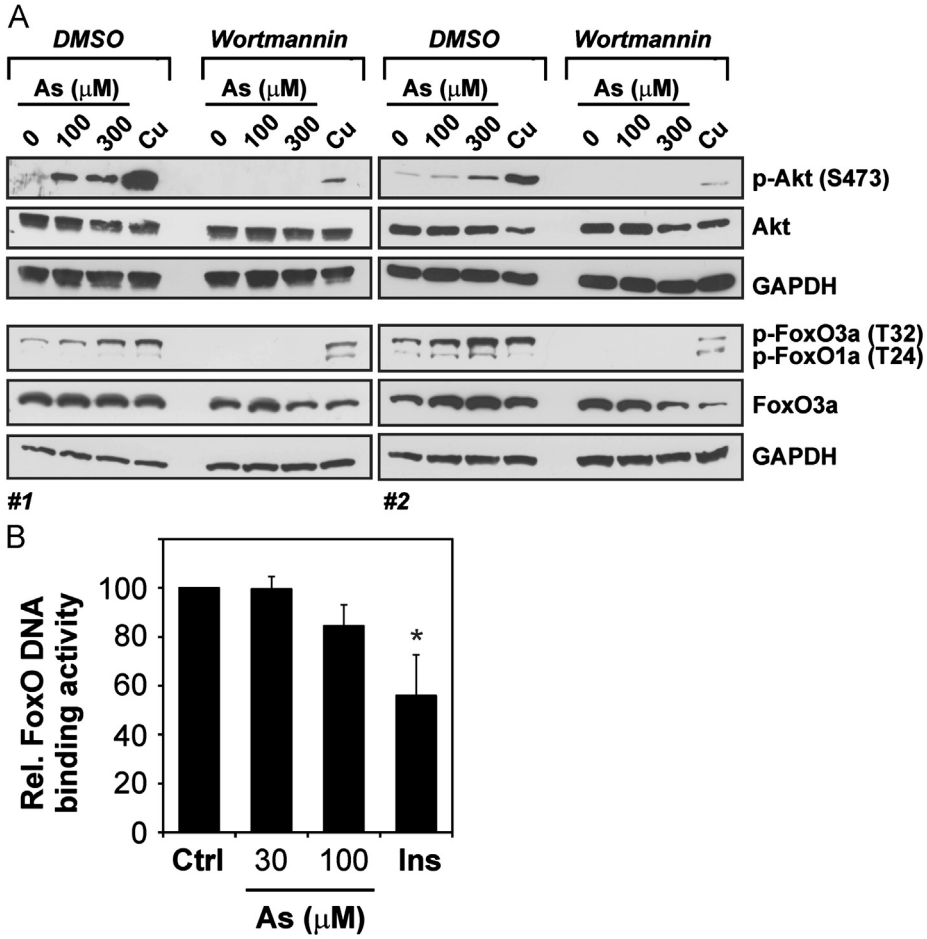

Fig. 4. *FoxO and Akt phosphorylation: role of PI3K and consequences*. (A) HaCaT human keratinocytes were grown to near confluence, then held in serum-free medium overnight, followed by incubation with 200 nM of the PI3K inhibitor wortmannin for 30 min in Hanks' balanced salt solution (HBSS). Cells were washed with PBS and incubated with arsenite (100 and 300 μM) or copper sulfate (10 μM) in HBSS for another 60 min prior to lysis and Western blotting analysis of phosphorylation of Akt and FoxO1a/3a. Two independent sets of experiments are shown. (B) HaCaT cells were grown to near confluence, held in serum-free medium overnight, followed by incubation with arsenite (30 and 100 μM) or insulin (100 nM) in HBSS for 60 min. Relative FoxO-DNA binding activity in nuclear extracts of exposed cells was determined in an ELISA-based transcription factor DNA binding assay. Data are given as means of 3 independent experiments ± SD. *Significantly different from control ($P < 0.05$; ANOVA, Dunnett's post-test). The decrease induced by arsenite is marginally significant ($P < 0.1$).

Interaction with crucial thiols: a mechanism for arsenite to modulate FoxOs?

Of all divalent transition metal ions tested so far, copper ions appear to be the most efficient stimulators of FoxO phosphorylation and inactivation, followed by Zn^{2+} [5,6,27,28]. As exposure to copper ions induces the cellular generation of ROS [29], it may be hypothesized that ROS are important mediators in metal-induced FoxO modulation. However, considering the fact that redox-inactive Zn ions inactivate FoxOs in a fashion similar to copper, and redox-active Fe(II) does not [28], a different mechanism of action appears more likely.

Here, copper ions again showed a much higher potential than arsenite to induce FoxO and Akt phosphorylation, effective already at 10 μM (Figs. 2 and 3). Nevertheless, the strong stimulation of FoxO phosphorylation induced by arsenite is evident.

Like copper and zinc ions, arsenite and other trivalent arsenicals strongly interact with sulfur ligands; in particular, it was demonstrated that arsenite may form adducts with peptides through interaction with cysteine thiol(ate)s [30–32]. Therefore, it is conceivable that regulators of the PI3K/Akt cascade such as protein tyrosine phosphatases (PTPases), which are exquisitely sensitive toward thiol reagents due to their active site cysteine [33], are potential arsenite targets. PTPase inactivation would cause a net stimulation of the controlled signaling cascade: indeed, arsenite metabolites (mono- and di-methylarsenite) were shown to be potent inhibitors of cellular PTPase activity, although arsenite per se only weakly interacted with isolated PTPases [34]. Moreover, PTEN (phosphatase and tensin homolog on chromosome 10), a PTPase-like lipid phosphatase controlling PI3K/Akt signaling was demonstrated to be inactivated (in a fashion reversed by dithiothreitol) in cardiomyocytes exposed to arsenic trioxide [35]. Other potential targets of arsenite in our setting include Ser/Thr phosphatases such as calcineurin, which is known to dephosphorylate Akt and to be sensitive to oxidative stimuli [36]. Low micromolar concentrations of arsenite were in fact shown to lower calcineurin activity in HaCaT cells [37]. The exact arsenite target in HaCaT cells that triggers stimulation of FoxO phosphorylation and FoxO inactivation upon interaction with arsenite remains to be identified.

Conclusions

Exposure of HaCaT cells to arsenite results in FoxO phosphorylation (Fig. 3) and inactivation (Fig. 4B). Inactivation of FoxOs and the concomitant decrease in transcriptional regulation is anticipated to result in a decreased production of proteins involved in the control of proliferation and of apoptosis [11]. Although in general, therefore, FoxOs are regarded as tumor suppressors [11], it remains to be determined in how far the FoxO phosphorylation/inactivation observed in this study contributes to As-induced carcinogenesis.

Contributions of authors

IH performed experiments. IH and LOK jointly conceived the study, analyzed data, wrote manuscript.

Conflict of interest statement

There is no conflict of interest to be declared.

Acknowledgments

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