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Acute and long-term effects of arsenite in HepG2 cells: modulation of insulin signaling

Ingrid Hamann · Kerstin Petroll · Xiaoqing Hou · Anwar Anwar-Mohamed · Ayman O. S. El-Kadi · Lars-Oliver Klotz

Abstract Epidemiological studies have indicated a relationship between the prevalence of diabetes and exposure to arsenic. Mechanisms by which arsenic may cause this diabetogenic effect are largely unknown. The phosphoinositide 3′-kinase (PI3K)/Akt signaling pathway plays an important role in insulin signaling by controlling glucose metabolism, in part through regulating the activity of FoxO transcription factors. The present study aimed at investigating the effect of short and long-term exposure to arsenite on insulin signaling in HepG2 human hepatoma cells, the role of PI3K/Akt signaling therein and the modulation of target genes controlled by insulin. Exposure of cells to arsenite for 24 h rendered cells less responsive toward stimulation of Akt by insulin. At the same time, short-term exposure to arsenite induced a concentration-dependent increase in phosphorylation of Akt at Ser-473, followed by phosphorylation of FoxO proteins at sites known to be phosphorylated by Akt. Phosphorylation of FoxOs was prevented by wortmannin, pointing to the involvement of PI3K. Arsenite exposure resulted in attenuation of FoxO DNA binding and in nuclear exclusion of FoxO1a-EGFP. A 24-h exposure of HepG2 cells to submicromolar concentrations of arsenite resulted in downregulation of glucose 6-phosphatase (G6Pase) and selenoprotein P (SelP) mRNA levels. Curiously, arsenite had a dual effect on SelP protein levels, inducing a small increase in the nanomolar and a distinct decrease in the micromolar concentration range. Interestingly, arsenite-induced long-term effects on G6Pase and SelP mRNA or SelP protein levels were not blocked by the PI3K inhibitor, wortmannin. In conclusion, arsenite perturbs cellular signaling pathways involved in fuel metabolism: it impairs cellular responsiveness toward insulin, while at the same time stimulating insulin-like signaling to attenuate the expression of genes involved in glucose metabolism and the release of the hepatokine SelP, which is known to modulate peripheral insulin sensitivity.

Keywords Insulin signaling · FoxO transcription factors · Arsenic · Akt · HepG2 cells · Selenium homeostasis

Introduction

Arsenic is a known human carcinogen and has been classified as a group 1 carcinogen by the International
Agency for Research on Cancer (IARC) (IARC 2004). Beyond being carcinogenic, however, there is strong evidence for diabetogenic effects of arsenic, i.e. for a link between arsenic in drinking water and the development of diabetes mellitus (DM) in humans. Epidemiological studies have established a link between As in drinking water (>150 µg/l, i.e. approx. 2 µM) and an elevated incidence of DM in affected populations [see (Maull et al. 2012) for a recent comprehensive review]; a connection between arsenic, diet and obesity/diabetes has also been drawn in animal studies (Paul et al. 2011), and various aspects of insulin signaling were demonstrated in cell culture to be affected by exposure to arsenic compounds.

A major signaling cascade stimulated by insulin in target cells is the phosphoinositide 3'-kinase (PI3K)-dependent activation of the Ser/Thr kinase Akt, which results in phosphorylation of several target molecules, such as phosphodiesterase 3B [resulting in modulation of cAMP levels (Kitamura et al. 1999)], glycogen synthase kinase-3 [GSK3; stimulating glycogen formation in response to high glucose levels (Frame and Cohen 2001)], and it mediates GLUT4 translocation to the cell membrane [to allow for glucose uptake by target cells (Zaid et al. 2008)]. In addition to these immediate effects on carbohydrate metabolism, insulin modulates gene expression, for example through regulation of transcription factors of the forkhead box, class O (FoxO) group (Barthel et al. 2005).

FoxO transcription factors (with three isoforms, FoxO1a, 3a and 4, expressed in most human cells) are key regulators of the expression of genes involved in fuel metabolism [e.g., glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK)], but also in the control of proliferation and cell cycle regulation (e.g., p27Kip and GADD45a), as well as antioxidant defense [e.g., manganese superoxide dismutase (MnSOD) and catalase] (Monsalve and Olmos 2011). Activity and subcellular localization of FoxO factors is regulated by Akt, which phosphorylates FoxO proteins, resulting in their inactivation and nuclear exclusion (Monsalve and Olmos 2011). FoxO inactivation results in abrogation of expression of FoxO target genes, such as those coding for the aforementioned key proteins in gluconeogenesis, G6Pase and PEPCK.

Insulin stimulates hepatic glycogen synthesis via Akt-dependent phosphorylation and inactivation of GSK3, while insulin-induced Akt-dependent phosphorylation, inactivation and nuclear export of FoxO transcription factors results in downregulation of hepatic glucose production through gluconeogenesis. Under conditions of insulin resistance, which is a hallmark of type 2 DM, stimulation and regulation of these hepatic events maintaining stable glucose plasma levels are disturbed (Barthel et al. 2005).

Recent studies have shown an impairment of insulin-induced Akt phosphorylation/activation, GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes exposed to arsenic compounds (Paul et al. 2007; Xue et al. 2011). However, the mechanisms underlying these effects have not been fully elucidated yet.

Here, we investigate the effect of arsenite on insulin-induced stimulation of the insulin signaling cascade, focusing on the activity of the insulin receptor and of downstream targets such as Akt and FoxO proteins in HepG2 human hepatoma cells. In addition, we investigate the immediate effect of exposure to arsenite on insulin signaling, focusing on FoxO transcription factors and the expression of selected FoxO target genes, including those coding for G6Pase and for selenoprotein P (SelP) (Walter et al. 2008).

SelP is the major plasma selenoprotein. It is of hepatic origin and serves the transport of selenium from the liver to peripheral tissues (Burk and Hill 2005). SelP is a hepatokine that was demonstrated to impair insulin sensitivity in target cells (Misu et al. 2010). Recently, epidemiological studies indicated a potential link between plasma selenium levels and type 2 DM [see Rayman and Stranges (2013) for a recent comprehensive review].

We find that arsenite thoroughly perturbs insulin signaling in liver cells, not only impairing but also strongly imitating insulin action. In addition, we describe a potential link between arsenic and selenium homeostasis through the modulation of SelP expression.

**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) and insulin stock solutions (0.1 mM in water) were both aliquoted and held at −20 °C; sodium arsenite and sodium selenite were held as a stock solution of 100 mM in water, copper sulfate as a stock solution of 10 mM in water and kept at 4 °C. Linsitinib was purchased from SelleckChem (Houston, TX, USA), diluted in DMSO to a stock solution of 10 mM, aliquoted and stored at −80 °C.

Cell culture and treatment of cells

HepG2 human hepatoma cells were obtained from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany) and held at 37 °C in a humidified atmosphere with 5 % (v/v) CO2 and 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).

Prior to all 60 min treatments, HepG2 cells were grown for 24 h and then held in serum-free medium for another 24 h, washed once with PBS, followed by incubation in the presence of arsenite, copper sulfate or insulin diluted into HBSS. For experiments with wortmannin (used at a final concentration of 200 nM), cells were pre-incubated with the inhibitor for 30 min in HBSS, followed by washing cells once with PBS and exposure to arsenite or copper ions in HBSS (without wortmannin). For experiments with linsitinib (used at a final concentration of 1 μM), cells were pre-incubated with the inhibitor for 60 min in serum-free DMEM, followed by washing cells once with PBS and exposure to arsenite or insulin in HBSS in the continued presence of linsitinib. For all incubation steps with wortmannin or linsitinib, DMSO was used as vehicle control.

For all 24 h treatments, HepG2 cells were treated with arsenite in serum-free medium with incubation commencing 24 h after seeding. For experiments with wortmannin, cells were pre-incubated with the inhibitor (at a final concentration of 100 nM) for 60 min in serum-free DMEM, followed by exposure to arsenite or insulin in the continued presence of wortmannin, which, due to its instability in cell culture medium and the need to inhibit any newly formed PI3K, was added another four times during the 24 h treatment (at 100 nM for each addition) with arsenite or insulin.

Cell viability

After exposure to arsenite for 1 or 24 h, cells were washed with PBS and incubated in serum-free cell culture medium for another 24 h. Cell viabilities were assessed by incubating and staining viable cells with neutral red (final concentration: 66 mg/l in DMEM) at 37 °C for 2 h. Cells were carefully washed twice with PBS, and neutral red incorporated by cells was extracted 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). Neutral red contents of cells held in the absence of arsenite were considered as indicating 100 % viability.

Determination of glutathione and glutathione disulfide

Glutathione (GSH) and glutathione disulfide (GSSG) were determined enzymatically according to (Anderson 1985), with minor modifications (Abdelmohsen et al. 2003). 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 at 4 °C. Total glutathione (GSH plus GSSG) and, after blocking thiols with 2-vinylpyridine, GSSG were determined from supernatants using 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of NADPH and glutathione reductase (Anderson 1985).

Western blotting

For analysis of InsR, IGF1R, Akt, FoxO1a, FoxO3a, GSK-3α, GAPDH and β-actin 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) bromphenol blue, pH 6.8] after treatment, followed by brief sonication and heating at 95 °C for 5 min. For detection of SelP, cell
culture supernatants were collected in pre-chilled tubes and centrifuged at 20,000 x g for 5 min at 4 °C to remove cell debris. Aliquots of the supernatants were mixed with 1/4 volume of 4x Laemmli buffer resulting in a final concentration of 62.5 mM Tris/HCl, 2 % (w/v) SDS, 10 % glycerol, 50 mM dithiothreitol and 0.01 % (w/v) bromophenol blue, pH 6.8. With supernatants collected, cells were lysed in 1 % SDS and used for protein determination in a bicinchoninic acid (BCA)-based protein assay (Pierce/Thermo Scientific, Rockford, USA). SelP contents in the supernatants were normalized over protein amounts in the corresponding cell extracts.

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-phospho-Akt (S473), anti-phospho-InsR/IGF1R (Tyr-1150,1151/Tyr-1135,1136), anti-phospho-GSK-3α antibodies were from Cell Signaling Technology (New England Biolabs, Pickering, ON, Canada); Murine and chicken anti-GAPDH antibodies from Millipore (Billerica, MA, USA) were used. The murine anti-β-actin antibody was from Sigma-Aldrich (Oakville, ON, Canada), the goat anti-SelP antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were from GE Healthcare (Mississauga, ON, Canada). HRP-conjugated anti-chicken IgG was from Millipore (Billerica, MA, USA); HRP-conjugated anti-goat IgG from Santa Cruz Biotechnology (Dallas, TX, USA). Incubations 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 was in 5 % (w/v) non-fat dry milk in TBST.

FoxO1a localization

FoxO1a localization was analyzed by fluorescence microscopy. Cells were grown to approximately 70 % confluence and were transfected with 3 μg of FoxO1a-EGFP plasmids using Nanofectin transfection reagent (PAA) according to the manufacturer’s instructions. The FoxO1a-EGFP expression plasmid (Kortylewski et al. 2003) was kindly provided by Dr. Andreas Barthel (Endokrinologikum, Bochum, Germany). Fluorescence microscopy of cells expressing EGFP-tagged FoxO1a was performed on an Axio Observer A1 fluorescence microscope (Zeiss, Toronto, ON, Canada). Analysis of EGFP-positive cells was done by counting and separating cells into three categories with respect to the major localization of FoxO1a-EGFP (nuclear, cytosolic or both).

FoxO1a DNA binding

FoxO1a DNA binding activity was assessed 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 (Active Motif, Carlsbad, CA, USA) 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.

Immunoprecipitation

After treatment, cells were washed once with PBS and lysed in 500 μl RIPA buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris–HCl (pH 8), 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 1 mM DTT), followed by brief sonication. Insoluble material was removed by centrifugation for 10 min at 14,000 g and 4 °C. Protein concentration in supernatants was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific), and equal amounts of protein (between 500 and 900 μg, depending on the experiment) from each lysate were incubated with 2 μg of precipitating antibody overnight at 4 °C [rabbit polyclonal anti-IR-β (Santa Cruz Biotechnology) or rabbit monoclonal anti-IR-β (Cell Signaling)]. Immune complexes were precipitated with protein G magnetic beads (Life Technologies) or protein A/G agarose beads (Santa Cruz Biotechnology), separated from the lysate and washed three times in RIPA buffer.
Magnetic or agarose beads were resuspended in 2× Laemmli buffer, heat-denatured, centrifuged and supernatants separated by SDS-PAGE on a 10% polyacrylamide gel, transferred to nitrocellulose membranes and analyzed for tyrosine phosphorylation using the “4G10 Platinum” monoclonal anti-phosphotyrosine antibody (Millipore, Billerica, MA, USA). Immunoprecipitation was controlled for by reprobing membranes with anti-IR-β antibody. Membrane blocking was in 5% (w/v) BSA in TBST, all primary antibody incubations were in 1% (w/v) BSA in TBST and all secondary antibody incubations were in TBST.

Real time RT-PCR analyses

Total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Burlington, ON, Canada) according to the manufacturer’s instructions. 1 μg of RNA was reverse-transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies, Burlington, ON, Canada) according to the manufacturer’s instructions. Quantitation of SelP, G6Pase, p27Kip and HPRT mRNA levels were performed by real-time PCR, employing specific TaqMan® gene expression assays (Applied Biosystems, Life Technologies, Burlington, ON, Canada) using probes 5’-labeled with 6-FAM and 3’ with MGBNFQ (Minor groove binder/Non-fluorescent quencher). The following TaqMan gene expression assays were employed: SelP (Hs01032845_m1), G6Pase catalytic subunit (Hs00609178_m1), p27Kip (Hs01597588_m1) and, as an internal control, HPRT1 (Hs01003267_m1). Real time PCR analyses were performed in the ABI Prism 7500 system (Applied Biosystems) using a PCR protocol according to the manufacturer’s instructions.

Results and discussion

In order to investigate the effects of arsenite exposure on insulin signaling, we chose HepG2 human hepatoma cells, a well-described insulin-responsive cell culture model for the analysis of hepatic insulin effects (Podskalny et al. 1985). For our analyses of acute and long-term arsenite effects on insulin signaling in these cells, we chose subcytotoxic concentrations of up to 1,000 μM (acute) and of no more than 10 μM (long-term), respectively: Whereas a 1 h-exposure of HepG2 cells to arsenite in concentrations as high as 1 mM caused no significant loss in cell viability 24 h post-exposure, a 24 h exposure to 30 μM or more decreased viability significantly (Fig. 1a). The ability of arsenic to induce oxidative stress in exposed cells is well established: arsenic was demonstrated to support the formation of ROS intracellularly, eliciting...
oxidative damage to DNA, lipids and proteins (Jomova and Valko 2011; Schwerdtle et al. 2003). In line with this—although significant only at As concentrations as high as 1 mM—a loss in cellular glutathione (GSH) was observed in HepG2 cells exposed to arsenite for 60 min (Fig. 1b). Interestingly, this loss in glutathione did not coincide with significantly increased levels of oxidized glutathione (data not shown), pointing to the induction of other mechanisms of GSH depletion, such as GSH utilization in the formation of arsenic-GSH conjugates or through glutathiolation of proteins (Flora 2011; Leslie 2012).

Arsenite lowers insulin sensitivity of HepG2 cells

Arsenic has previously been demonstrated to attenuate insulin-induced glucose uptake in 3T3-L1 adipocytes (Walton et al. 2004) which was suggested to be due to an interference with the phosphoinositide-dependent protein kinase (PDK)-catalyzed phosphorylation of Akt (Paul et al. 2007).

In order to examine whether this loss of insulin sensitivity is also induced in liver cells and to test whether that translates to various levels in insulin signaling, we analyzed the phosphorylation status of several crucial proteins in the insulin cascade, from insulin receptor (InsR) to Akt and Akt substrates, including GSK3 and insulin-regulated FoxO transcription factors.

Using phospho-specific antibodies, we analyzed an InsR tyrosine residue cluster that is required to be phosphorylated for full activation of the receptor upon stimulation and that contains Tyr-1150 and Tyr-1151 (numbers referring to the short, InsR-A isoform, corresponding to tyrosines 1162 and 1163 in InsR-B). These sites correspond to Tyr-1135/Tyr-1136 in the related IGF1R, whose phosphorylation would be detected by the same antibody.

Stimulation of HepG2 cells with insulin induced a strong phosphorylation of these tyrosines, as well as a strong phosphorylation of proteins downstream of the InsR/IGF1R (Fig. 2a, b): As expected, the serine/threonine kinase Akt was phosphorylated at Ser-473, indicative of its being activated; two known Akt substrates, glycogen synthase kinase-3 (GSK3) and FoxO transcription factors, were phosphorylated at Akt target sites contributing to inactivation of these proteins (Fig. 2).

In cells exposed to arsenite prior to insulin treatment, insulin signaling was impaired (Fig. 2). While insulin-induced phosphorylation of the InsR/IGF1R at Tyr-1150/1151 and Tyr-1135/1136 was attenuated in cells pre-treated with 10 μM arsenite, induction of Akt phosphorylation at Ser-473 by insulin was drastically impaired regardless of arsenite concentration or insulin treatment duration.

GSK3 and FoxO proteins are direct substrates of Akt, and their phosphorylation by Akt results in their inactivation. Using phospho-specific antibodies, we analyzed for phosphorylation of Akt target sites of GSK3α (Ser-21) and FoxO1α/FoxO3α (Thr-24/Thr-32) (Fig. 2). 10 μM arsenite caused a distinct inhibition of insulin-induced FoxO phosphorylation detectable after 5 min of insulin treatment. In contrast, this effect was no longer detectable with increased duration of insulin treatment. For GSK3α, no general attenuation of insulin-induced phosphorylation by arsenite was detected; however, GSK3 phosphorylation over control levels following insulin stimulation was strongly impaired due to the elevation of GSK3 basal phosphorylation by arsenite pretreatment (Fig. 2b, compare 0 min values for GSK3). In contrast, arsenite did not cause a basal phosphorylation of FoxO proteins, Akt or InsR (Fig. 2b, compare 0 min values for FoxO, Akt and InsR).

The strong effect of arsenite on the activity of Akt compared to the rather modest effect on the FoxO proteins and GSK3 was quite surprising. However, insulin-induced Akt phosphorylation levels recovered distinctly when increasing the treatment time with insulin to 30 min, reaching about 80 % of the phosphorylation signal in the respective control in HepG2 cells exposed to 3 μM arsenite and about 50 % in those exposed to 10 μM (data not shown). Apparently, insulin signaling is delayed, rather than abrogated.

Arsenite as an insulin mimetic (I): stimulation of Akt/FoxO signaling and the role of InsR

Several metal ions can induce insulin-like signaling processes, and both Akt and FoxO were demonstrated to be phosphorylated in human keratinocytes exposed to arsenite (Hamann and Klotz 2013). Thus, we next tested for an insulin-mimetic action of As in HepG2 cells, focusing on FoxO transcription factors which are known to be regulated by insulin and to control
expression of several genes, including p27Kip, G6Pase and SelP.

Both Akt and FoxO were strongly phosphorylated in a concentration-dependent manner in HepG2 cells exposed to arsenite for 60 min (Fig. 3). Insulin and copper ions, which had previously been demonstrated to strongly stimulate Akt (Ostrakhovitch et al. 2002) were chosen as positive controls, and phosphorylation of both proteins was induced by both stimuli (Fig. 3).

Using wortmannin, an inhibitor of phosphoinositide 3′-kinases, we tested for a role of PI3K in arsenite-induced FoxO phosphorylation. As shown in Fig. 4, FoxO phosphorylation was attenuated in cells pretreated with wortmannin, indicating that PI3K is required for arsenite-induced FoxO phosphorylation.

Arsenite-induced phosphorylation of Akt and FoxO: role of insulin receptor

The PI3K/Akt pathway is typically activated via stimulation of receptor tyrosine kinases, e.g. the insulin-responsive receptors, InsR or IGF1R. To test if the observed PI3K-dependent phosphorylation of Akt and FoxO is due to a stimulation of InsR or IGF1R, we first analyzed phosphorylation of Tyr-1150,-1151 and/or Tyr-1135,-1136, Akt phosphorylation at Ser-473, phosphorylation of FoxO1a/3a at Thr-24/-32 or phosphorylation of glycogen synthase kinase-3α (GSK3α) at Ser-21. a The blots shown are representative of three independent experiments with similar results. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) staining was used to demonstrate equal loading of gels. b Densitometric analysis of phosphorylation signals relative to GAPDH. Values for controls at 20 min insulin were set equal to 1. Data are means of 3 independent experiments ±SEM.
tyrosine residues might be phosphorylated upon exposure of cells to arsenite for 60 min, we performed immunoprecipitation of the insulin receptor, followed by Western blotting analysis of general tyrosine phosphorylation with an anti-phospho-tyrosine antibody: only little, if any, arsenite-induced tyrosine phosphorylation of the insulin receptor was detectable, whereas insulin expectedly caused a significant tyrosine phosphorylation of its receptor (Fig. 5b). In order to further substantiate these data, we also used a different (monoclonal) antibody for precipitation of IR, followed by phospho-tyrosine detection—with essentially the same result: no tyrosine phosphorylation of the IR was elicited by exposure to arsenite, whereas insulin stimulated substantial tyrosine phosphorylation of its receptor (data not shown). In summary, only very minor tyrosine phosphorylation of InsR was elicited by exposure to arsenite at concentrations that caused strong Akt and FoxO phosphorylation.

At the same time, we found that exposure to arsenite does cause a significant increase in overall tyrosine phosphorylation in exposed cells (Fig. 5c), suggesting that, while not significantly enhancing InsR/IGF1R tyrosine phosphorylation, arsenite does stimulate tyrosine phosphorylation in general.

Moreover, we found arsenite-induced Akt/FoxO phosphorylation to be independent of InsR/IGF1R tyrosine kinase activity: employing linsitinib (OSI-906), a dual InsR/IGF1R inhibitor (Mulvihill et al. 2009), we tested for a role of the InsR/IGF1R in arsenite-induced Akt and FoxO phosphorylation. As shown in Fig. 5d, both basal and insulin-induced InsR and IGF1R phosphorylation was blunted in cells pre-

Fig. 3 Phosphorylation of Akt and FoxO in cells exposed to arsenite. HepG2 human hepatoma cells were grown for 24 h, held in serum-free cell culture medium for another 24 h, then washed with PBS and exposed to the indicated concentrations of sodium arsenite, 10 μM copper sulfate or 100 nM insulin in HBSS for 60 min. Akt phosphorylation at Ser-473 (a) or phosphorylation of FoxO1a and FoxO3a at Thr-24 and Thr-32 (b), respectively, were analyzed by Western blotting and immunodetection using phosphospecific antibodies. The blots shown are representative of 3 independent experiments with similar results. GAPDH staining was used to demonstrate equal loading of gels. For densitometric analyses of Akt or FoxO phosphorylation, values were normalized using GAPDH signals. Controls were set equal to 1. Data are means of 3 independent experiments ±SEM

Fig. 4 Role of phosphoinositide 3′-kinase in arsenite-induced Akt and FoxO phosphorylation. HepG2 human hepatoma cells were grown for 24 h, held in serum-free cell culture medium for another 24 h, washed with PBS, 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 exposed to 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 FoxO1a/3a. One of 3 independent sets of experiments with similar result is shown
treated with linsitinib. Likewise, insulin-induced Akt and FoxO phosphorylation was blocked in the presence of linsitinib. In sharp contrast to insulin-treated cells, however, neither the phosphorylation of Akt nor of FoxO were affected in cells exposed to arsenite (Fig. 5d), implying that arsenite-induced phosphorylation of these proteins is independent of InsR/IGF1R.

In addition, an interference of arsenite with receptor tyrosine kinases other than InsR or IGF1R appears unlikely as a cause of Akt activation since neither the phosphorylation of Akt nor of FoxO were affected by the presence of genistein, a general tyrosine kinase inhibitor (data not shown).

Arsenite as an insulin mimetic (II): inactivation and nuclear exclusion of FoxO transcription factors

Akt-dependent phosphorylation of FoxO proteins, as induced by insulin, results in their inactivation and

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**Fig. 5** Phosphorylation of Akt and FoxO in cells exposed to arsenite: Role of insulin receptor. a HepG2 human hepatoma cells were grown for 24 h, held in serum-free cell culture medium for another 24 h, then washed with PBS and exposed to the given concentrations of sodium arsenite or 100 nM insulin in HBSS for 60 min. InsR and IGF1R phosphorylation at Tyr-1150,-1151 and Tyr-1135,-1136, respectively, were detected using a phosphospecific antibody. Densitometric analysis of InsR/IGF1R phosphorylation signals was normalized over GAPDH levels. Controls were set equal to 1. Data are means of 3 independent experiments ± SEM. Data significantly different from control (ANOVA with Dunnett’s post-test) are indicated by asterisks (**P < 0.01). b HepG2 cells were grown as above and exposed to the given concentrations of sodium arsenite or 100 nM insulin in HBSS for 60 min. Extracts were prepared, followed by immunoprecipitation (IP) of the InsR using an antibody recognizing the InsR β subunit, followed by immunodetection (ID) of general tyrosine phosphorylation. c Immunodetection of general tyrosine phosphorylation in cells exposed to arsenite for 60 min by Western blotting. d HepG2 cells were grown as above, followed by incubation with 1 µM of the InsR inhibitor linsitinib for 60 min in serum-free medium. Cells were washed with PBS and incubated with arsenite (300 or 1,000 µM) or insulin (100 nM) in HBSS in the continued presence of linsitinib for another 60 min prior to lysis and Western blotting analysis of phosphorylation of InsR/IGF1R, Akt or FoxO1a/3a. One of three independent sets of experiments with similar result is shown. All blots shown are representative of 3 independent experiments with similar results.

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nuclear exclusion. To examine whether arsenite causes such inactivation of FoxO transcription factors, we analyzed its DNA binding activity and subcellular distribution.

We analyzed FoxO binding to its DNA target sequence employing an ELISA-based FoxO DNA binding assay. In line with observed FoxO phosphorylation occurring upon exposure of cells to insulin or arsenite for 60 min, endogenous FoxO-DNA binding activity was significantly attenuated under these conditions (Fig. 6a).

We then analyzed changes in subcellular localization of FoxO in response to arsenite. HepG2 human hepatoma cells were transiently transfected with a plasmid coding for a FoxO1a-EGFP fusion protein for another 24 h in serum-free medium. Cells were washed with PBS followed by incubation with arsenite (10–1,000 µM), copper sulfate (10 µM) or insulin (100 nM) in HBSS for 60 min (b, c) and with arsenite (3-10 µM), or insulin (100 nM) in serum-free DMEM for 24 h (d), respectively. The subcellular distribution of FoxO1a-EGFP was analyzed by fluorescence microscopy and numbers of cells with predominantly nuclear or predominantly cytosolic FoxO1a-EGFP determined. All data are given as means of 3 independent experiments ± SEM. Asterisks indicate significant difference from control (*P < 0.05; **P < 0.01, ANOVA, Dunnett’s post-test).

Quantitation of these effects was performed by counting cells with predominantly nuclear vs. cytoplasmic accumulation of FoxO1a-EGFP. Arsenite at 100 µM elicited the same effect.

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We then analyzed changes in subcellular localization of FoxO in response to arsenite. HepG2 human hepatoma cells were transiently transfected with a plasmid coding for an EGFP-tagged version of FoxO1a. Transfected cells were exposed to insulin or copper (as positive controls) or arsenite for 60 min. Subcellular localization of FoxO1a-EGFP was then analyzed microscopically. As shown in Fig. 6b, FoxO1a-EGFP was found in all parts of transfected cells under control conditions, whereas both insulin and copper ions stimulated a strong nuclear exclusion and cytoplasmic accumulation of FoxO1a-EGFP. Arsenite at 100 µM elicited the same effect.

Quantitation of these effects was performed by counting cells with predominantly nuclear vs. cytoplasmic localization of FoxO1a-EGFP (Fig. 6c). Under basal conditions, FoxO1a-EGFP was predominantly nuclear in roughly 20 % of all cells analyzed, whereas less than 10 % had the protein exclusively cytosolic. Approximately 70 % of the cells had both nuclear and cytoplasmic FoxO1a-EGFP. The numbers of cells with nuclear and cytosolic FoxO1a-EGFP were set equal to 1 for control conditions and changes upon exposure to insulin/copper or arsenite investigated. As expected, and in line with causing Akt-dependent FoxO phosphorylation, insulin and copper ions stimulated nuclear exclusion of FoxO1a proteins, resulting in a decrease in relative numbers of cells carrying FoxO1a-EGFP predominantly in the nucleus (black bars) and an increase in numbers of cells with cytosolic FoxO1a-EGFP (white bars; Fig. 6c). Treatment with arsenite for 60 min at 100 µM and above induced significant FoxO1a-EGFP nuclear exclusion, thus imitating insulin (Fig. 6c).
We then analyzed in how far the effects on FoxO subcellular localization identified under acute As exposure conditions persist for longer periods of time. We therefore exposed cells to low arsenite concentrations (3 and 10 μM, not impairing cell viabilities) for 24 h, followed by analysis of subcellular distribution of FoxO1a-EGFP. As depicted in Fig. 6d, the relative numbers of cells with nuclear or cytoplasmic FoxO1a-EGFP were no longer as dramatically different from control conditions as seen under acute exposure (see Fig. 6c)—both for arsenite and insulin treatment. Of note, 10 μM arsenite induced an increase in numbers of cells with cytoplasmic FoxO1a-EGFP that was similar in extent to the effect observed with insulin (Fig. 6d) and likely recruited from the pool of cells harboring the overexpressed protein in both nucleus and cytoplasm (not shown in the bar graphs) rather than those that have the protein predominantly nuclear.

Thus, whereas high doses of arsenite were required to imitate insulin under acute exposure conditions (Fig. 6c), low concentrations of arsenite achieved this effect under long-term exposure (Fig. 6d).

The observed long-term arsenite effect was rather unexpected, considering the lack of detectable changes in FoxO phosphorylation under these conditions (Fig. 2a, compare 0 min values for phospho-FoxO). We believe that the functional assay using FoxO1a-EGFP is slightly more sensitive and better suited to detect changes in FoxO activity than Western blotting as in Fig. 2—despite its limitations of (i) requiring a step of actual counting and grouping of cells, and thus a component of subjectivity in the analysis and (ii) despite requiring overexpression of a tagged protein.

Insulin-like modulation of gene expression by arsenite

Arsenite clearly induced FoxO phosphorylation, FoxO deactivation, the nuclear exclusion of FoxO after 60 min and FoxO cytoplasmic accumulation after 24 h treatment. We then tested whether arsenite also affects the expression of genes known to be regulated by the FoxO transcription factors and downregulated by insulin, such as the genes coding for the cell cycle inhibitor p27Kip (Machida et al. 2003), the gluconeogenesis enzyme glucose 6-phosphatase (G6Pase) (Schmoll et al. 1996) and the hepatokine selenoprotein P (SelP) (Speckmann et al. 2008; Walter et al. 2008). In line with its insulin imitating effect, exposure of HepG2 cells to arsenite for 24 h drastically lowered G6Pase and SelP mRNA levels, while slightly decreasing p27Kip levels (Fig. 7a–c). Effects on G6Pase mRNA were significant already at submicromolar arsenite concentrations.

Downregulation of the expression of a FoxO target gene would be in line with PI3K/Akt signaling being stimulated, leading to the phosphorylation and inactivation of FoxO transcription factors. Therefore, we next tested whether inhibition of PI3K attenuated arsenite-induced downregulation of FoxO target genes. HepG2 cells were pretreated with wortmannin, an irreversible inhibitor of phosphoinositide 3′-kinases, followed by exposure to arsenite for 24 h. Insulin was used as a positive control in this experiment, known to downregulate FoxO-dependent gene expression via stimulation of PI3K and Akt. As shown in Fig. 7d, e, wortmannin indeed attenuated insulin-induced downregulation of G6Pase mRNA levels and, albeit to a lesser extent, of SelP mRNA levels. Wortmannin also increased basal G6Pase levels (Fig. 7d, inset: here wortmannin control was normalized against DMSO control treatment), suggesting that G6Pase mRNA levels respond to changes in basal PI3K activity already. Indeed, no such basal wortmannin effect was observed with SelP (data not shown).

In contrast to insulin, arsenite-induced down-regulation of G6Pase and SelP mRNA levels was not only much stronger than insulin-induced effects, but was not counteracted by wortmannin (Fig. 7d, e). This suggests that down-regulation of G6Pase and SelP gene expression by arsenite is not mediated by PI3K, different from the acute effects observed at higher arsenite concentrations (see Fig. 4).

In order to test whether the modulation of mRNA levels also translates into protein synthesis, we tested for SelP production by cells exposed to arsenite for 24 h. SelP is the major selenium containing protein in plasma, serving as selenium transporter from liver to extrahepatic tissue (Burk and Hill 2005) and recently identified as a hepatokine that confers insulin resistance to peripheral tissues (Misu et al. 2010). As SelP is secreted by hepatocytes, we collected cell culture supernatants from HepG2 cells held in culture medium with added sodium selenite (200 nM) (Fig. 8a) or culture medium without extra selenite added (Fig. 8b). SelP contents detected by Western blotting were normalized over protein content of the cells producing SelP.
Fig. 7 Arsenite attenuates expression of FoxO target genes independently of PI3K. a–c HepG2 human hepatoma cells were grown for 24 h and treated with 0.1–10 µM arsenite in serum-free medium for 24 h. d, e HepG2 cells were pre-treated with 100 nM wortmannin or DMSO in serum-free medium for 1 h, followed by an incubation with 0.3–10 µM arsenite or 100 nM insulin in serum-free medium for 24 h, during which 100 nM fresh wortmannin or DMSO (control) was added 4 times. RNA was isolated, followed by quantitative RT-PCR analyses of levels of p27 Kip mRNA (a), glucose 6-phosphatase mRNA (b, d) or selenoprotein P mRNA (c, e), which were normalized against hypoxanthine/guanine phosphoribosyl transferase (HPRT1) mRNA levels. All data are given as means of 3 (a, b, c) or 4 (d, e) independent experiments ±SEM. Asterisks indicate values significantly different from control (*P < 0.05; **P < 0.01, ANOVA, Dunnett’s post-test). Pound signs indicate significant difference of each wortmannin-treated sample from respective DMSO-treated sample (#P < 0.05, unpaired t test). Data were normalized against respective (DMSO or wortmannin) controls. Inset wortmannin control relative to DMSO control.

Fig. 8 Modulation of selenoprotein P levels by arsenite. HepG2 human hepatoma cells were grown for 24 h and held in serum-free cell culture medium for another 24 h in the presence (a) or in the absence (b) of added 0.2 µM selenite. Cells were washed with PBS and exposed to 0.1–10 µM arsenite in serum-free cell culture medium with (a) or without (b) added 0.2 µM selenite. Cell culture supernatants were submitted to Western blot analyses. Selenoprotein P (SelP) signals were densitometrically analyzed and normalized against the protein amount in corresponding cell culture extracts. All data are given as means of 3 independent experiments ± SEM. Data significantly different from control (ANOVA with Dunnett’s post-test) are indicated by asterisks (*P < 0.05, **P < 0.01).
Interestingly, arsenite both up- and down-regulated SelP production, depending on whether selenium had been added. In the presence of added selenium, arsenite significantly decreased SelP production at 3 and 10 µM arsenite (Fig. 8a)—which is in line with the effects observed with mRNA levels (Fig. 7c). Without selenium added, we found a biphasic effect of arsenite on the production of SelP, with a small but significant increase in the nanomolar and a decrease in the micromolar concentration range (Fig. 8b).

Next we tested if inhibition of PI3K with wortmannin affects SelP release from HepG2 cells. Similar to our observation with G6Pase mRNA levels, wortmannin increased basal SelP protein production (data not shown), suggesting that wortmannin concentrations employed were effective. Interestingly, however, the inhibitory effect of arsenite on SelP production was no longer observed in control cells exposed to DMSO solvent control. Accordingly, no attenuation of arsenite effects by wortmannin was observable. We hypothesize that the experimental procedure might have affected the results at the protein level: not only was the final DMSO concentration rather high (0.4 % v/v), but the cell culture dishes also had to be removed from the incubator four times to add wortmannin or DMSO to the cell medium (see Materials and Methods). We addressed these concerns by performing the experiment adding wortmannin only once (i.e. 1 h prior to arsenite exposure), followed by arsenite treatment in the presence of fresh wortmannin for 24 h. Here, cumulative wortmannin concentrations added with or after arsenite were 100 nM (rather than 400 nM) and DMSO present at 0.1 % only. A clear inhibitory effect of arsenite on SelP release was observed at 10 µM (similar to Fig. 8a), while no attenuating effect of wortmannin could be seen (data not shown). Again, arsenite-induced inhibition of SelP protein production appears to be PI3K-independent.

In summary, arsenite exposure caused a downregulation of G6Pase mRNA and SelP mRNA and protein production. None of these appears to be mediated by PI3K—different from the previously described acute effects of arsenite (Fig. 4).

Conclusions

We have demonstrated here that arsenite may not only antagonize but also imitate insulin signaling in human hepatoma cells. While strongly stimulating insulin-like signaling events in hepatoma cells by interfering with the signaling cascade at a level not directly linked to the InsR (Fig. 5), these same cells are less sensitive to stimulation by the actual hormone (Fig. 2). Whether or not there is a link between the observed stimulatory effect of arsenite and insulin resistance in these cells, remains to be established. However, it is clear from our data that arsenite thoroughly perturbs insulin signaling in HepG2 cells, suggesting that it will interfere with endogenous control and regulation of fuel metabolism.

Regarding the mode of the observed FoxO modulation upon short-term exposure to arsenite, FoxO activity may be modulated by various stressful stimuli, including reactive oxygen species (ROS), such as hydrogen peroxide (Bartholome et al. 2010; Essers et al. 2005; Kops et al. 2002). As arsenite may cause the cellular formation of ROS (Ruiz-Ramos et al. 2009), one might conclude that ROS mediate the effect of arsenite on FoxO transcription factors. However, exposure of HepG2 cells to hydrogen peroxide resulted in detectable activation of Akt and FoxO phosphorylation only at H₂O₂ concentrations above 10 mM (data not shown)—a concentration beyond those anticipated to be generated in cells exposed to applied arsenite concentrations. We therefore believe that arsenite-induced Akt activation is independent of the generation of ROS but due to arsenite/thiol interactions: Like copper and zinc ions (Barthel et al. 2007; Eckers et al. 2009; Kroencke and Klotz 2009; Walter et al. 2006), arsenite and other trivalent arsenicals strongly interact with sulfur ligands; in particular, arsenite may form adducts with peptides through interaction with cysteine thiol(ate)s (Kitchin and Wallace 2005, 2006; Watanabe and Hirano 2012). Therefore, it is conceivable that regulators of the PI3K/Akt cascade that are sensitive toward thiol reagents—such as protein tyrosine phosphatases (PTPases), which harbor an active site cysteine (Östman et al. 2011)—are potential arsenite targets. PTPase inactivation would result in a net stimulation of the signaling cascade under their control: indeed, arsenite metabolites were shown to be potent inhibitors of cellular PTPase activity, although arsenite per se only weakly interacted with isolated PTPases (Rehman et al. 2012). As Akt activation upon arsenite exposure was independent of InsR/IGF1R stimulation in HepG2 cells (Fig. 5), it
is unlikely that (a) PTPase(s) regulating InsR tyrosine phosphorylation (such as PTP-1B) is/are major mediators of arsenite-induced Akt activation. Rather, the target molecule is suggested to be downstream of the insulin/IGF1 receptor tyrosine kinase. PTEN (phosphatase and tensin homolog on chromosome 10), a PTPase-like lipid phosphatase that regulates PI3K/Akt signaling by catalyzing the dephosphorylation of 3’-phosphoinositides, was demonstrated to be reversibly inactivated in cardiomyocytes exposed to arsenic trioxide (Wan et al. 2011). Other potential targets of arsenite in our setting include Ser/Thr phosphatases that dephosphorylate Akt and are sensitive to oxidative stimuli. One example is calcineurin (Sommer et al. 2002), whose activity was indeed found to be lowered in cells exposed to low micromolar concentrations of arsenite (Musson et al. 2012). The exact molecular target of arsenite in HepG2 cells that triggers FoxO inactivation upon interaction with arsenite remains to be identified.

Besides decreasing insulin sensitivity (Fig. 2), arsenite seems to contribute to a disturbance of glucose metabolism by affecting the transcription of genes involved in gluconeogenesis and insulin resistance. We have seen a dramatic decrease in mRNA levels of the gluconeogenesis enzyme, G6Pase (Fig. 7), and an attenuation of the production of SelP (Figs. 7, 8), a protein whose biosynthesis was previously demonstrated to be regulated like that of G6Pase (Speckmann et al. 2008) and to confer insulin resistance to insulin target tissues (Misu et al. 2010). Interestingly, the effects of arsenite on G6Pase and SelP mRNA levels appear not to be due to activation of PI3K (Figs. 7d, e). The exact mechanism of arsenite-induced attenuation of G6Pase and SelP expression remains to be elucidated.

Similar to arsenite (Fig. 2), selenocompounds interfere with insulin-induced signaling (Pinto et al. 2011), and it was postulated that this may occur via modulation of cellular redox homeostasis (Pinto et al. 2011; Steinbrenner et al. 2011), e.g. through the stimulated production of antioxidant selenoenzymes whose activity alters ROS levels in cells. A confounding effect of arsenic exposure in studies linking higher selenium levels to an increased risk of type 2 DM was indeed discussed recently (Rayman and Stranges 2013), although the authors then went on to discard that idea. Irrespective of its significance for DM, the finding that arsenite modulates SelP expression may have implications for selenium homeostasis in general, as distribution of selenium to extrahepatic tissues is suggested to be downstream of the insulin/IGF1 receptor tyrosine kinase. PTEN (phosphatase and tensin homolog on chromosome 10), a PTPase-like lipid phosphatase that regulates PI3K/Akt signaling by catalyzing the dephosphorylation of 3’-phosphoinositides, was demonstrated to be reversibly inactivated in cardiomyocytes exposed to arsenic trioxide (Wan et al. 2011). Other potential targets of arsenite in our setting include Ser/Thr phosphatases that dephosphorylate Akt and are sensitive to oxidative stimuli. One example is calcineurin (Sommer et al. 2002), whose activity was indeed found to be lowered in cells exposed to low micromolar concentrations of arsenite (Musson et al. 2012). The exact molecular target of arsenite in HepG2 cells that triggers FoxO inactivation upon interaction with arsenite remains to be identified.

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Of note, the regulation of SelP expression by FoxOs occurs in concert with hepatocyte nuclear factor (HNF) 4α (Speckmann et al. 2008), which was reported recently to be downregulated by chronic exposure of HepG2 cells to arsenite (Pastoret et al. 2013). The authors put the downregulation of HNF1α and HNF4α identified as a response of arsenite exposure in HepG2 cells into a carcinogenesis context. It will be of interest to further investigate a possible link between arsenic-induced carcinogenesis and the impaired distribution of selenium to tissues in potentially chemopreventive form [for a recent review on selenium in chemoprevention, see Steinbrenner et al. (2013)] to extrahepatic tissues. It remains to be determined in how far FoxO proteins and selenoprotein P levels are affected by exposure to arsenite in vivo and in how far they are involved in arsenic-induced pathogenesis.

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