Molecular Mechanisms of the Diabetogenic Effects of Arsenic: Inhibition of Insulin Signaling by Arsenite and Methylarsonous Acid

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BACKGROUND: Increased prevalences of diabetes mellitus have been reported among individuals chronically exposed to inorganic arsenic (iAs). However, the mechanisms underlying the diabetogenic effects of iAs have not been characterized. We have previously shown that trivalent metabolites of iAs, arsenite (iAsIII) and methylarsonic acid (MAsIII) inhibit insulin-stimulated glucose uptake (ISGU) in 3T3-L1 adipocytes by suppressing the insulin-dependent phosphorylation of protein kinase B (PKB/Akt).

OBJECTIVES: Our goal was to identify the molecular mechanisms responsible for the suppression of PKB/Akt phosphorylation by iAsIII and MAsIII.

METHODS: The effects of iAsIII and MAsIII on components of the insulin-activated signal transduction pathway that regulate PKB/Akt phosphorylation were examined in 3T3-L1 adipocytes.

RESULTS: Subtoxic concentrations of iAsIII or MAsIII had little or no effect on the activity of phosphatidylinositol 3-kinase (PI-3K), which synthesizes phosphatidylinositol-3,4,5-triphosphate (PIP3), or on phosphorylation of PTEN (phosphatase and tensin homolog deleted on chromosome ten), a PT3 phosphatase. Neither iAsIII nor MAsIII interfered with the phosphorylation of 3-phosphoinositide-dependent kinase-1 (PDK-1) located downstream from PI-3K. However, PDK-1 activity was inhibited by both iAsIII and MAsIII. Consistent with these findings, PDK-1-catalyzed phosphorylation of PKB/Akt (Thr308) and PKB/Akt activity were suppressed in exposed cells. In addition, PKB/Akt (Ser473) phosphorylation, which is catalyzed by a putative PDK-2, was also suppressed. Notably, expression of constitutively active PKB/Akt restored the normal ISGU pattern in adipocytes treated with either iAsIII or MAsIII.

CONCLUSIONS: These results suggest that inhibition of the PDK-1/PKB/Akt-mediated transduction step is the key mechanism for the inhibition of ISGU in adipocytes exposed to iAsIII or MAsIII, and possibly for impaired glucose tolerance associated with human exposures to iAs.

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Arsenic (As) is a naturally occurring toxic metalloid and a potent human carcinogen [International Agency for Research on Cancer (IARC) 1987]. The cancer-promoting effects of environmental exposures to inorganic arsenic (iAs) have been examined by epidemiologic studies and in laboratory experiments. Much less attention has been paid to the adverse effects of iAs that do not involve malignancies. Epidemiologic evidence suggests that type 2 (noninsulin dependent) diabetes mellitus may be one of the most common noncancerous diseases associated with chronic exposures to iAs. Increased prevalences of type 2 diabetes or symptoms consistent with this disease have been associated with the consumption of drinking water containing high levels of iAs (Chen et al. 1995; Lit et al. 1994; Rahman et al. 1998, 1999; Tseng et al. 2000, 2002; Wang et al. 2003) or with chronic exposures to iAs in occupational settings (Jensen and Hansen 1998; Rahman and Axelson 1995; Rahman et al. 1996). Although not all epidemiologic studies support the association between iAs exposure and diabetes (Navas-Acien et al. 2006), the existing evidence provides sufficient basis for investigation of the diabetogenic effects of iAs.

Type 2 diabetes is characterized by disruptions in whole-body glucose homeostasis due to insulin resistance and impaired glucose utilization by peripheral tissues, including skeletal muscle and adipose tissue. The insulin-dependent activation of glucose uptake in these tissues is one of the key mechanisms that regulates glucose homeostasis. The insulin-activated signal transduction mechanism that stimulates glucose uptake by adipocytes has been extensively studied. It includes the autophosphorylation of the β-subunit of the insulin receptor (IRβ) upon binding of insulin to the α-subunit of the receptor (IRα), the subsequent tyrosine phosphorylation of insulin receptor substrate 1 or 2 (IRS-1 or -2), and the binding of a phosphorylated IRS (p-IRS) to the regulatory (p85) subunit of the class IA phosphatidylinositol 3-kinase (PI-3K) that leads to the activation of its catalytic (p110) subunit. The activated PI-3K catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) at the plasma membrane to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Farese 2001; Ruderman et al. 1990; White et al. 2002). PIP3 facilitates 3-phosphoinositide-dependent kinase-1/2 (PKB/Akt) and two atypical protein kinase C (PKCζ) and PKCλζ) at the plasma membrane to phosphatidylinositol-3,4,5-triphosphate (PIP3) (Farese 2001; Ruderman et al. 1990). The activation of PKB/Akt induces the translocation and fusion of GLUT4-containing vesicles with the plasma membrane, as well as the insulin-independent activation of glucose uptake (ISGU). Notably, the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The authors declare they have no competing financial interests. Received 30 October 2006; accepted 29 January 2007.
Inhibition of insulin signal by inorganic and methyl arsenic

et al. 1999) and to inhibit the cytoskeletal protein synthesis in Swiss 3T3 mouse cells (Li and Chou 1992).

The mechanisms by which exposure to iAs may induce impaired glucose tolerance have not been systematically studied. Data on the effects of As on glucose homeostasis have been generated almost exclusively in studies that examined the metabolism of nutrients under severe stress induced by chemical or physical stimuli. Results of in vitro studies have consistently shown significant increases in basal (insulin-independent) glucose uptake by various types of cells or dissected tissues exposed to cytotoxic concentrations of a trivalent iAs, arsenite (iAsIII), or an aromatic derivative of AsIII, phenylarsine oxide (PAO) (Bazaine et al. 2003, 2004; Brazy et al. 1980; McDowell et al. 1997; Pasternak et al. 1991; Short 1965; Sviderskaya et al. 1996; Widnell et al. 1990). Consistent with these findings, some in vivo studies have reported moderate or severe hypoglycemia in animals chronically exposed to toxic, often lethal, concentrations of iAsIII or arsenate (iAsV), in drinking water (Hughes and Thompson 1996; Pal and Chatterjee 1992).

cytotoxic and apoptotic effects of arsenicals. To determine cell viability, we used the MTT assay, which measures the conversion of 3-4,5-dimethylthiazol-2-yl-2,5-diphenytriazolium bromide (MTT) to purple formazan by mitochondrial dehydrogenases of viable cells (Carmichael et al. 1987), as previously described (Walton et al. 2004). Caspase-3 activity was examined in an assay mixture containing cell lysate and aminomethylcoumarin (AMC)-derived substrate, Z-DEVD-AMC (Molecular Probes, Carlsbad, CA). Cleavage of Z-DEVD-AMC by caspase-3 yields a blue-fluorescent product (excitation/emission wavelength = 342/411 nm) that was quantified by an HTS 7000 Bio Assay Reader (Perkin-Elmer, Norwalk, CT). We examined DNA fragmentation in adipocytes using TUNEL (terminal deoxynucleotidyltransferase-mediated nick end labeling). For this assay, adipocytes were cultured on glass coverslips coated with poly-L-lysine (Sigma Chemical Co.) and treated with arsenicals. Cells were then fixed in 4% buffered paraformaldehyde and permeabilized in a solution of 0.1% Triton X-100 and 0.1% sodium citrate (Sigma Chemical Co.). DNA strand breaks were enzymatically labeled with 3'-OH termini with fluorescein-linked nucleotides, using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Nuclei of both normal and apoptotic cells were stained with 100 nM 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co.). Labeled cells were visualized using a Nikon Microphot FXA fluorescent microscope (Nikon, Tokyo, Japan).

**Materials and Methods**

**Cell culture and treatment.** We obtained 3T3-L1 preadipocytes from Y. Patel (University of North Carolina, Greensboro, North Carolina). Myr-PKB/Akt–3T3-L1 preadipocytes expressing constitutively active PKB/Akt lacking the pleckstrin homology (PH) domain were provided by S. Summers (University of Colorado at Boulder, Boulder, Colorado). Addition of the src myristoylation sequence promotes constitutive membrane association and activation of PKB/Akt (Kohn et al. 1996a, 1996b). A2myr-PKB/Akt–3T3-L1 adipocytes, which express PKB/Akt containing a nonfunctional src myristoylation domain, and 3T3-L1 adipocytes containing the empty expression vector were also provided by S. Summers. All cell lines were cultured in Dulbecco’s modified Eagle medium (Gibco, Grand Island, NY) with high glucose, 10% fetal bovine serum (HyClone, Logan, UT), penicillin, and streptomycin (Sigma Chemical Co.). Cells were cultured at 37°C in a humidified incubator in a 90% air and 10% CO2 atmosphere. To induce differentiation, postconfluent cells were treated with a mixture of 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, and 1 µg/mL insulin (all from Sigma Chemical Co.) for 48 hr and cultured in insulin-containing medium for an additional 48 hr (Paul et al. 2003). All experiments were performed between days 9 and 12 postinduction, when more than 90% of cells were fully differentiated. Differentiated adipocytes were treated with iAsIII (sodium salt, Sigma Chemical Co.) or methylarsine oxide (provided by W. Cullen, University of British Columbia, Vancouver, Canada). Identity and purity of methylarsine oxide was confirmed by 1H-NMR and mass spectrometry. In aqueous solutions, methylarsine oxide is hydrolyzed to form MA3III (Petrick et al. 2001). Fresh stock solutions of iAsIII and MA3III in sterile phosphate-buffered saline (PBS) were prepared before each experiment to minimize the oxidation of iAsIII to iAsV or MA3III to methylarsonic acid (MA3V). Adipocytes were incubated with arsenicals or vehicle in a cell culture incubator for 4 hr.

**Glucose uptake assay.** The glucose uptake assay followed the previously described procedures (Paul et al. 2003). Briefly, adipocytes were serum starved in the presence or absence of arsenicals for 4 hr, washed with Krebs-Ringer phosphate (KRP) buffer, and treated with 1 µM insulin at 37°C for 10 min. Insulin-activated cells were incubated for 10 min with 200 µM 2-[(34-C)]deoxy-D-glucose (0.1 µCi/well) (NEN Life Science Products, Inc., Boston, MA). To measure basal (insulin-independent) glucose uptake, we incubated cells with radiolabeled glucose without pretreatment with insulin. After the incubation, cells were washed twice with PBS (0°C), and lysed in a solution of 0.5 N NaOH and 10% SDS. Radioactivity in cell lysates was measured, using a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

**Evaluation of cytotoxic and apoptotic effects of arsenicals.** To determine cell viability, we used the MTT assay, which measures the conversion of 3-4,5-dimethylthiazol-2-yl-2,5-diphenytriazolium bromide (MTT) to purple formazan by mitochondrial dehydrogenases of viable cells (Carmichael et al. 1987), as previously described (Walton et al. 2004). Caspase-3 activity was examined in an assay mixture containing cell lysate and aminomethylcoumarin (AMC)-derived substrate, Z-DEVD-AMC (Molecular Probes, Carlsbad, CA). Cleavage of Z-DEVD-AMC by caspase-3 yields a blue-fluorescent product (excitation/emission wavelength = 342/411 nm) that was quantified by an HTS 7000 Bio Assay Reader (Perkin-Elmer, Norwalk, CT). We examined DNA fragmentation in adipocytes using TUNEL (terminal deoxynucleotidyltransferase-mediated nick end labeling). For this assay, adipocytes were cultured on glass coverslips coated with poly-L-lysine (Sigma Chemical Co.) and treated with arsenicals. Cells were then fixed in 4% buffered paraformaldehyde and permeabilized in a solution of 0.1% Triton X-100 and 0.1% sodium citrate (Sigma Chemical Co.). DNA strand breaks were enzymatically labeled with 3’-OH termini with fluorescein-linked nucleotides, using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Nuclei of both normal and apoptotic cells were stained with 100 nM 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co.). Labeled cells were visualized using a Nikon Microphot FXA fluorescent microscope (Nikon, Tokyo, Japan).

**Immunofluorescent analysis of GLUT4.** Adipocyte cultures on glass cover slips were treated with arsenicals, activated with insulin, and incubated with D-glucose (Sigma Chemical Co.). After fixation with 4% buffered paraformaldehyde, adipocytes were rinsed with ice-cold PBS and incubated with poly-L-lysine (0.5 mg/mL) for 1 min. Cells were then treated
with a hypotonic buffer [10 mM HEPES (pH 7.5), 2 mM MgCl₂, 23 mM KCl, 1 mM EDTA] and pulse sonicated for 5 sec in a sonication buffer [30 mM HEPES (pH 7.5), 6 mM MgCl₂, 70 mM KCl, 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], using a Fisher Model 100 Sonic Dismembrator equipped with a 12.7 × 0.3-cm probe (Fisher Scientific, Hampton, NH). Plasma membrane sheets were captured, using a Zeiss LSM 110 (Carl Zeiss Microscopy, Santa Cruz, CA) and labeled with Alexa-633 anti-GLUT4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa-488 anti-β-actin (Santa Cruz Biotechnology). For a single assay we used the immunoprecipitate from cells cultured in one 10-cm plate. The assay conditions were as follows:

**PI-3K assay.** PI-3K was immunoprecipitated from control (untreated) adipocytes or from adipocytes exposed to arsenicals with an anti-phosphotyrosine (PY20) antibody. PI-3K immunoprecipitated from insulin-activated adipocytes pretreated with 1 nM wortmannin (a specific inhibitor of PI-3K) was used as a negative control. The enzyme activity was measured in a 50-µL assay mixture containing the immunoprecipitated PI-3K, 20 mM HEPES (pH 7.4), 50 mM MgCl₂, 200 mM adenosine, 40 mM adenosine 5'-tri-phosphate (ATP) (all from Sigma Chemical Co.), 20 µCi [γ-32P]-ATP (NEN Life Science Products, Inc.), and 1-µM-phosphatidylinositol (PI) (Avanti Polar Lipids Inc., Alabaster, AL) as a substrate (Augustine et al. 1991). The reaction was stopped by 1 N HCl. A 30-min incubation at 37°C of the assay mixture containing PI-3K from control cells resulted in the formation of radiolabeled phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP₂). To simplify the analysis, a 10-min incubation that yielded only PIP was used.

**Immunoblot analyses.** Protein extracts were prepared from cells treated with arsenicals, activated with insulin, and incubated with D-glucose, using a 25-mM HEPES (pH 7.4) lysis buffer containing 1% NP 40, 100 mM NaCl, 2% glycerol, 5 mM sodium fluoride, 1 mM EDTA, 1 mM sodium orthovanadate (Na₃VO₄), 1 mM sodium pyrophosphate, 1 mM PMSF, 40 µg/mL aprotinin, 20 µg/mL leupeptin, and 20 µg/mL pepstatin (all from Sigma Chemical Co.). Protein extracts were separated by 10% SDS-PAGE, electroblotted to Immobilon-P membranes (Millipore, Burlington, MA), and probed using the following antibodies: anti-p38 MAPK (mitogen-activated protein kinase), anti-p-p38 MAPK, anti-PKB/Akt, anti-p-PKB/Akt(Ser473) and anti-p-PKB/Akt(Thr308), anti-PTEN (phosphatase and tensin homolog deleted on chromosome 10), anti-p-PTEN(Ser380), anti-p-PDK1-Ser241) (Cell Signaling Technology, Beverly, MA); anti-PI-3K(p85) (Upstate Biotechnology, Lake Placid, NY); and anti-β-actin (Abcam, Cambridge, MA). The antigen-antibody complexes on immunoblots were treated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using autoradiography or the Gene Gnome imaging system (Syngene, Frederick, MD).

**Protein kinase activity assays.** We measured PI-3K, PKB/Akt, and PDK-1 activities in cell lysates from adipocytes treated with arsenicals and activated with insulin after immunoprecipitation with specific antibodies bound to protein G agarose beads (Santa Cruz Biotechnology). For a single assay we used the immunoprecipitate from cells cultured in one 10-cm plate. The assay conditions were as follows:

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Results of the cell viability, glucose uptake, and protein kinase activity assays were evaluated by analysis of variance with Tukey multiple comparison posttest using a GraphPad Instat statistical software package (GraphPad Software, San Diego, CA). Differences among means with $p < 0.05$ were considered statistically significant.

Results

Our previous work has shown that trivalent arsenicals inhibit ISGU by 3T3-L1 adipocytes. However, the possible association between the inhibition of ISGU and a general loss of cell functions due to the cytotoxicity of arsenicals has not been thoroughly examined. In this study we examined ISGU and cell viability in adipocytes exposed for 4 hr to iAsIII or MAsIII at a wide range of concentrations. Consistent with our previous report (Walton et al. 2004), stimulation of 3T3-L1 adipocytes with insulin increased glucose uptake by 9- to 11-fold over basal levels (data not shown). ISGU was significantly inhibited by concentrations as low as 5 µM iAsIII and 0.5 µM MAsIII (Figure 1A, B). In contrast, cell viability decreased only when concentrations of iAsIII and MAsIII exceeded 1 mM and 5 µM, respectively. Gross abnormalities in adipocyte morphology were absent at all concentrations tested. Although minor cell detachment did occur at higher concentrations (> 200 µM iAsIII and ≥ 10 µM MAsIII). The estimated IC50 (concentration that results in the inhibition of ISGU by 50%) values for the inhibition of ISGU were 25 µM for iAsIII and 4 µM for MAsIII. In comparison, the LC50 (concentration that results in a decrease of cell viability by 50%) values characterizing the cytotoxic effects were 11 mM for iAsIII and 15 µM for MAsIII. Thus, the inhibition of ISGU by iAsIII and MAsIII at or below IC50 values was not due to impaired adipocyte viability. However, both iAsIII and MAsIII can induce cell apoptosis (Lau et al. 2004; McCollum et al. 2005; Namgung and Xia 2001). At early stages, apoptotic processes may affect cell functions without having

Figure 3. Four-hour exposures to subtoxic concentrations of iAsIII or MAsIII do not increase DNA fragmentation in cultured adipocytes. DNA fragmentation was measured by TUNEL in 3T3-L1 adipocytes treated with 50 µM iAsIII or 2 µM MAsIII for 4, 24, 48, and 72 hr. Untreated adipocytes were used as controls. Color images show green fluorescein signal of fragmented DNA in apoptotic cells. Gray-scale images illustrate the corresponding cell morphology. Representative fields of two independent experiments are shown. Bars = 40 µm.
immediate effects on cell viability. We examined apoptotic markers in adipocytes exposed for 4 hr to 50 µM iAsIII and 2 µM MAsIII, the concentrations that effectively inhibit ISGU, but are far below the minimal cytotoxic concentrations. Under these exposure conditions, both iAsIII and MAsIII significantly increased the activity of caspase-3, an early marker of apoptosis (Figure 2A). Adipocytes treated with 500 µM H2O2 were used as positive controls for this experiment. Pretreatment with 75 µM Ac-Asp-Glu-Val-Asp-CHO (AC-DEVD-CHO), a cell-permeable caspase-3 inhibitor, prevented caspase-3 activation by both arsenicals and by hydrogen peroxide. However, pretreatment with AC-DEVD-CHO did not prevent the decrease in ISGU in cells treated with either iAsIII or MAsIII (Figure 2B), suggesting that the inhibition of ISGU was independent of processes associated with early stages of apoptosis. TUNEL was used to determine the degree of DNA fragmentation in adipocytes exposed to 50 µM iAsIII or 2 µM MAsIII (Figure 3). Adipocyte nuclei were stained with DAPI to determine the total number of cells (data not shown). The average apoptotic index (percentage of TUNEL-positive cells) was about 16% for control adipocytes and did not change after a 4-hr exposure to either iAsIII or MAsIII. However, the apoptotic index increased considerably after longer exposure times, reaching an average of 32% for iAsIII and 39% for MAsIII after 24 hr and more than 90% after 72-hr exposure to either arsenical. These data suggest that 4-hr exposures to 50 µM iAsIII or 2 µM MAsIII did not compromise cell viability or integrity. In addition, neither 50 µM iAsIII nor 2 µM MAsIII induced p38 MAPK phosphorylation during the 4-hr exposure (data not shown). Therefore, the inhibition of ISGU is not associated with stress and is likely due to specific effects of these arsenicals on mediators of insulin signaling or on the cellular components involved in glucose transport. Based on these results, 4-hr exposures to 50 µM iAsIII and 2 µM MAsIII were used in further experiments to examine the effects of iAsIII or MAsIII on components of the insulin-activated signal transduction pathway in 3T3-L1 adipocytes.

The effects of iAsIII or MAsIII on mediators of insulin signaling would ultimately depend on the intracellular concentrations and metabolic conversion of these arsenicals. We examined the distribution of As species in adipocytes after a 4-hr exposure to iAsIII or MAsIII, using HG-AAS. Cells exposed to 50 µM iAsIII retained about 3 times more As than cells exposed to 2 µM MAsIII (Figure 4). Retained As represented 2.5 and 16% of the total As in cultures exposed to iAsIII or MAsIII, respectively. Only iAs and MAs species were detected in adipocyte cultures exposed to iAsIII and MAsIII, respectively, indicating that no methylation conversion took place during the 4-hr exposures. These findings are consistent with previous reports that found adipocytes to be inefficient methylators of iAs (Walton et al. 2004).

The translocation of GLUT4 from the perinuclear compartment to the plasma membrane is a prerequisite for glucose uptake in adipocytes stimulated with insulin. We used immunofluorescent staining in this study to examine the association of GLUT4 with the plasma membranes of insulin-stimulated 3T3-L1 adipocytes treated with 50 µM iAsIII or 2 µM MAsIII for 4 hr and from control (untreated) cells that were or were not stimulated with insulin (Figure 5). Stimulation with insulin dramatically increased the GLUT4-specific fluorescent signal in plasma membrane lawns of control cells. GLUT4 signals in plasma membrane lawns isolated from insulin-stimulated cells treated with either iAsIII or MAsIII were noticeably weaker compared with control insulin-stimulated cells, suggesting that both arsenicals interfered with the translocation of GLUT4 in response to insulin stimulation.
The impaired ISGU in adipocytes exposed to trivalent arsenicals has previously been linked to the inhibition of components of the insulin signal transduction pathway located downstream of IRS1/2, but upstream of PKB/Akt (Walton et al. 2004). PI-3K is located downstream of IRS. The binding of p-IRS to the regulatory (p85) subunit of PI-3K in response to insulin stimulates the PI-3K-catalyzed production of PIP3 from PIP2. In this study, the association of p-IRS with PI-3K was examined in insulin-stimulated adipocytes exposed for 4 hr to 50 µM iAsIII or 2 µM MA3III. Neither iAsIII nor MA3III affected the amount of PI-3K (p85), immunoprecipitated with an anti-phospho-tyrosine (PY20) antibody, which reacts with phosphorylated tyrosine residues of IRS in the insulin-activated PI-3K complex (Figure 6A). PI-3K activity was measured in adipocytes exposed for 4 hr to 50 or 100 µM iAsIII or to 2 or 5 µM MA3III. Exposures to iAsIII had no effect on PI-3K activity. A relatively small decrease in PI-3K activity was detected in cells exposed to 2 µM MA3III; however, no changes were found in cells exposed to 5 µM MA3III (data not shown). Effects of MA3III on PI-3K activity were further analyzed in an in vitro assay mixture containing PI-3K immunoprecipitated from control insulin-stimulated adipocytes. Addition of MA3III into this mixture at concentrations up to 50 µM did not inhibit PI-3K activity (data not shown). PTEN, a PIP3 phosphatase, is involved in the regulation of PIP3 levels in adipocytes. PTEN activity is regulated by a casein kinase 2-catalyzed phosphorylation on its C-terminal non-catalytic regulatory domain, which includes Ser380 (Torres and Pulido 2001). Neither 50 µM iAsIII nor 2 µM MA3III altered the levels of total PTEN or pPTEN (Ser380) (Figure 6A). No changes in the ratio of phosphorylated pPTEN (Ser380) to total PTEN were found in insulin-stimulated adipocytes exposed to either iAsIII or MA3III (Figure 6B).

Phosphorylation on Ser241 is required for optimal activity of PDK-1, a downstream effector of PI-3K (Casamayor et al. 1999). Figure 7A shows that exposures to 50 µM iAsIII or 2 µM MA3III had no significant effects on the level of Ser241-phosphorylated PDK-1 in insulin-stimulated adipocytes. However, PDK-1 activity was significantly lower in cells exposed to either iAsIII or MA3III, 47% and 57% of that in control cells, respectively (Figure 7B).

In the insulin-activated signal transduction pathway, PKB/Akt is the downstream effector of PDK-1. The activation of PKB/Akt in response to insulin stimulation includes the phosphorylation of Ser473 and Thr308 residues (Toker and Newton 2000). Our previous work demonstrated that exposures to iAsIII or MA3III inhibit PKB/Akt phosphorylation on Ser473 (Walton et al. 2004), which is thought to be catalyzed by a putative Ser-kinase, PDK-2 (Toker and Newton 2000). PDK-1 is responsible for Thr308 phosphorylation, which is required for maximal PKB/Akt activity (Scheid et al. 2002). Immunoblot analysis carried out in this study showed that 4-hr exposures to 50 µM iAsIII or 2 µM MA3III inhibited the insulin-dependent phosphorylation of PKB/Akt on both Ser473 and Thr308 residues (Figure 8A). PKB/Akt activity in
insulin-stimulated adipocytes exposed to iAsIII and MAsIII was 47 and 28% of that in control insulin-activated cells, respectively (Figure 8B).

To further evaluate the role of the PDK-1/PKB/Akt signal transduction step as a target for trivalent arsenicals in the insulin-activated signal transduction pathway, we examined the effects of iAsIII or MAsIII on ISGU by adipocytes expressing constitutively active myr-PKB/Akt. Adipocytes expressing an inactive A2myr-PKB/Akt mutant or empty expression vector were used as negative controls. Consistent with the constitutive activation of PKB/Akt, glucose uptake by adipocytes expressing myr-PKB/Akt was elevated even in the absence of insulin stimulation (Figure 9). Four-hour exposures to 50 µM iAsIII or 2 µM MAsIII had no effect on ISGU by myr-PKB/Akt expressing cells. In contrast, both arsenicals inhibited ISGU in cells expressing the inactive A2myr-PKB/Akt mutant or the empty expression vector.

**Discussion**

Previous studies have shown that AsIII-containing species may affect glucose uptake by cultured cells or dissected tissues by two independent mechanisms that strictly depend on the concentration of AsIII. Highly-toxic concentrations of AsIII stimulate glucose uptake in the absence of insulin (Bazuine et al. 2003, 2004; Brazy et al. 1980; McDowell et al. 1997; Pasternak et al. 1991; Short 1965; Sviderskaya et al. 1996; Widnell et al. 1990) through a mechanism that involves activation of p38 MAPK-mediated stress signaling and PI-3K-dependent phosphorylation of PKB/Akt.

To further evaluate the role of the PDK-1/PKB/Akt signal transduction step as a target for trivalent arsenicals in the insulin-activated signal transduction pathway, we examined the effects of iAsIII or MAsIII on ISGU by adipocytes expressing constitutively active myr-PKB/Akt. Adipocytes expressing an inactive A2myr-PKB/Akt mutant or empty expression vector were used as negative controls. Consistent with the constitutive activation of PKB/Akt, glucose uptake by adipocytes expressing myr-PKB/Akt was elevated even in the absence of insulin stimulation (Figure 9). Four-hour exposures to 50 µM iAsIII or 2 µM MAsIII had no effect on ISGU by myr-PKB/Akt expressing cells. In contrast, both arsenicals inhibited ISGU in cells expressing the inactive A2myr-PKB/Akt mutant or the empty expression vector.

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Previous studies have shown that AsIII-containing species may affect glucose uptake by cultured cells or dissected tissues by two independent mechanisms that strictly depend on the concentration of AsIII. Highly-toxic concentrations of AsIII stimulate glucose uptake in the absence of insulin (Bazuine et al. 2003, 2004; Brazy et al. 1980; McDowell et al. 1997; Pasternak et al. 1991; Short 1965; Sviderskaya et al. 1996; Widnell et al. 1990) through a mechanism that involves activation of p38 MAPK-mediated stress signaling and PI-3K-dependent phosphorylation of PKB/Akt.

To further evaluate the role of the PDK-1/PKB/Akt signal transduction step as a target for trivalent arsenicals in the insulin-activated signal transduction pathway, we examined the effects of iAsIII or MAsIII on ISGU by adipocytes expressing constitutively active myr-PKB/Akt. Adipocytes expressing an inactive A2myr-PKB/Akt mutant or empty expression vector were used as negative controls. Consistent with the constitutive activation of PKB/Akt, glucose uptake by adipocytes expressing myr-PKB/Akt was elevated even in the absence of insulin stimulation (Figure 9). Four-hour exposures to 50 µM iAsIII or 2 µM MAsIII had no effect on ISGU by myr-PKB/Akt expressing cells. In contrast, both arsenicals inhibited ISGU in cells expressing the inactive A2myr-PKB/Akt mutant or the empty expression vector.
stable cyclic structure with two thiois, iAsIII may require three coordination bonds to form a stable enzyme-inhibitor complex. A lower affinity for binding to Cys21 and Cys23 may explain why iAsIII is a weaker inhibitor of PDK-1 than MAsIII. In addition, the difference in potencies of iAsIII and MAsIII to inhibit PDK-1 activity and ISGU may be due in part to differences in the uptake and/or retention of these arsenicals by adipocytes. Our data suggest that MAsIII was retained by 3T3-L1 adipocytes more efficiently than iAsIII. These findings are consistent with the results of previous studies in other cell types (Dopp et al. 2004; Drobna et al. 2005). Importantly, our data show that the expression of constitutively active myrPKB/Akt prevents the inhibition of ISGU by either iAsIII or MAsIII. These data provide further evidence that the inhibition of ISGU by 3T3-L1 adipocytes exposed to iAsIII and MAsIII is due to the inhibition of the PDK-1–catalyzed activation of PKB/Akt and that neither iAsIII nor MAsIII disrupts signal transduction steps downstream from PDK-1/PKB/Akt, or events associated with GLUT4 translocation to the plasma membrane.

In summary, subtoxic concentrations of iAsIII and MAsIII inhibit ISGU by 3T3-L1 adipocytes through a mechanism that involves the inhibition of PDK-1 activity and of PDK-1–catalyzed phosphorylation of PKB/Akt (Figure 10). The inhibition of ISGU by iAsIII and MAsIII, trivalent metabolites of iAs, is consistent with impaired glucose tolerance reported in individuals chronically exposed to iAs from the environment. In addition, the concentrations of iAsIII and MAsIII that inhibit ISGU by cultured adipocytes (as low as 5 and 0.5 µM, respectively) appear to be compatible with this type of exposure. Thus, taken together, this work provides a mechanistic basis for the diabetogenic effects of chronic environmental and occupational exposures to iAs.

References

Alessi DR, Deak M, Casamayor A, Caudwell FB, Mirror N, Norman DG, et al. 1997. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol 7:776–787. Altman RB, Libermann-Minotta CA, Luna-Lemus R, Calcapino M. 1989. Evidence for vicinal thiol and their functional role in glucosamine-6-phosphate deaminase from Escherichia coli. Arch Biochem Biophys 289:555–561. Augustine JA, Sutor SL, Abraham RT. 1991. Interleukin-2 and polyomavirus middle T antigen-induced modification of phosphatidylinositol 3-kinase activity in activated T lymphocytes. Mol Cell Biol 11:4421–4440. Bazuine MA, Mortarini F, Bieden RC, Houben RC, Maassen JA. 2004. Mitogen-activated protein kinase (MAPK) phosphatase-1 and -4 attenuate p38 MAPK during dexametha- sone-induced insulin resistance in 3T3-L1 adipocytes. Mol Endocrinol 18:1690–1707. Bazuine MA, Ouwens DM, Gomes de Mesquita DS, Maassen JA. 2003. Arsenite stimulated glucose transport in 3T3-L1 adipocytes involves both GLUT translocation and p38 MAPK activation. Eur J Biochem 270:3891–3893.
Rahman M, Tondel M, Chowdhury IA, Axelsson O. 1999. Relations between exposure to arsenic, skin lesions, and glucosuria. Occup Environ Med 56:277–281.

Rahman M, Wingren G, Axelsson O. 1996. Diabetes mellitus among Swedish art glass workers—an effect of arsenic exposure? Scand J Work Environ Health 22:146–149.

Ruderman NB, Kapeller R, White MF, Cantley LC. 1990. Activation of phosphatidylinositol 3-kinase by insulin. Proc Natl Acad Sci USA 87:1411–1415.

Scheid MP, Marignani PA, Woodgett JR. 2002. Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Mol Cell Biol 22:6247–6260.

Short AL, Wright, FE, Whitney JE. 1965. Effects of anaerobiosis and cell poisons on glucose uptake of hemidiaphragms and epididymal fat pads in vitro. Diabetes 14:128–131.

Souza K, Maddock DA, Zhang Q, Chen J, Chiu C, Mehta S, et al. 2001. Arsenite activation of PI3K/AKT cell survival pathway is mediated by p38 in cultured human keratinocytes. Mol Med 7:767–772.

Sowell MO, Robinson KA, Buse MG. 1988. Phenylarsine oxide and denervation effects on hormone-stimulated glucose transport. Am J Physiol 255:E159–E165.

Standaert ML, Galloway L, Karnam P, Bandypadhyay G, Moscat J, Farese RV. 1997. Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. J Biol Chem 272:30075–30082.

Sviderskaya EV, Jazrawi E, Baldwin SA, Widnell CC, Pasternak CA. 1996. Cellular stress causes accumulation of the glucose transporter at the surface of cells independently of their insulin sensitivity. J Membr Biol 149:133–140.

Tang Y, Powelka AM, Soriano NA, Czech MP, Guilherme A. 2005. PTEN, but not SHIP2, suppresses insulin signaling through the phosphatidylinositol 3-kinase/Akt pathway in 3T3-L1 adipocytes. J Biol Chem 280:22523–22529.

Tanti JF, Grillo S, Gremieux T, Coffer PJ, Van Obberghen E, Le Marchand-Brustel Y. 1997. Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes. Endocrinology 138:2005–2010.

Thomas DJ, Styblo M, Lin S. 2001. The cellular metabolism and systemic toxicity of arsenic. Toxicol Appl Pharmacol 176:127–144.

Toker A, Newton AC. 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. J Biol Chem 275:8271–8274.

Tolkacheva T, Chan AM. 2000. Inhibition of H-Ras transformation by the PTEN/MMAC1/TEP1 tumor suppressor gene. Oncogene 19:680–689.

Torres J, Pulido R. 2001. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to prostate-mediated degradation. J Biol Chem 276:983–998.

Tseng CH, Tai TY, Chong CK, Tseng CP, Lai MS, Lin BJ, et al. 2000. Long-term arsenic exposure and incidence of non-insulin-dependent diabetes mellitus: a cohort study in arseniasis-hyperendemic villages in Taiwan. Environ Health Perspect 108:847–851.

Tseng CH, Tseng CP, Chiou HY, Hsueh YM, Chong CK, Chen CJ. 2002. Epidemiologic evidence of diabetogenic effect of arsenic. Toxicol Lett 133:69–76.

Wada T, Sassaoka T, Funaki M, Hori H, Murakami S, Ishiki M, et al. 2001. Overexpression of SH2-Containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes via its 5’-phosphatase catalytic activity. Mol Cell Biol 21:1633–1646.

Walton FS, Harmon AW, Paul DS, Drobna Z, Patel YM, Styblo M. 2004. Inhibition of insulin-dependent glucose uptake by trivalent arsenicals: possible mechanism of arsenic-induced diabetes. Toxicol Appl Pharmacol 198:424–433.

Wang SL, Chiu JM, Chen CJ, Tseng CH, Chou WL, Wang CC, et al. 2003. Prevalence of non-insulin-dependent diabetes mellitus and related vascular diseases in southwestern arseniasis-endemic and nonendemic areas in Taiwan. Environ Health Perspect 111:155–159.

White MF, Kahan CR. 1994. The insulin signaling system. J Biol Chem 269:1–4.

Widnell CC, Baldwin SA, Davies A, Martin S, Pasternak CA. 1990. Cellular stress induces a redistribution of the glucose transporter. FASEB J 4:1604–1617.