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# Exposures to arsenite and methylarsonite produce insulin resistance and impair insulin-dependent glycogen metabolism in hepatocytes

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## Abstract

Environmental exposure to inorganic arsenic (iAs) has been shown to disturb glucose homeostasis, leading to diabetes. Previous laboratory studies have suggested several mechanisms that may underlie the diabetogenic effects of iAs exposure, including (i) inhibition of insulin signaling (leading to insulin resistance) in glucose metabolizing peripheral tissues, (ii) inhibition of insulin secretion by pancreatic β cells, and (iii) dysregulation of the methylation or expression of genes involved in maintenance of glucose or insulin metabolism and function. Published studies have also shown that acute or chronic iAs exposures may result in depletion of hepatic glycogen stores. However, effects of iAs on pathways and mechanisms that regulate glycogen metabolism in the liver have never been studied. The present study examined glycogen metabolism in primary murine hepatocytes exposed in vitro to arsenite ($iAs^{3+}$) or its methylated metabolite, methylarsonite ($MAs^{3+}$). The results show that 4-h exposures to $iAs^{3+}$ and $MAs^{3+}$ at concentrations as low as 0.5 and 0.2 pM, respectively, decreased glycogen content in insulin-stimulated hepatocytes by inhibiting insulin-dependent activation of glycogen synthase (GS) and by inducing activity of glycogen phosphorylase (GP). Further investigation revealed that both $iAs^{3+}$ and $MAs^{3+}$ inhibit insulin-dependent phosphorylation of protein kinase B/Akt, one of the mechanisms involved in the regulation of GS and GP by insulin. Thus, inhibition of insulin signaling (i.e., insulin resistance) is likely responsible for the dysregulation of glycogen metabolism in hepatocytes exposed to $iAs^{3+}$ and $MAs^{3+}$. This study provides novel information about the mechanisms by which iAs exposure impairs glucose homeostasis, pointing to hepatic metabolism of glycogen as one of the targets.

### Keywords

Arsenic exposure; Glycogen metabolism; Hepatocytes; Insulin resistance; Glycogen synthase

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## Introduction

A growing number of laboratory and epidemiological studies have demonstrated that chronic exposure to inorganic arsenic (iAs) is associated with an increased prevalence or incidence of diabetes mellitus (Navas-Acien et al. 2006; Maull et al. 2012; Sung et al. 2015). However, the molecular mechanisms that underlie the development of diabetes in the context of iAs exposure are not well understood. Previous laboratory studies using animal or in vitro models have identified several pathways of glucose metabolism and several regulatory mechanisms that are targeted by iAs or its metabolites. These studies have shown that trivalent iAs, arsenite ($iAs^{3+}$), and its methylated trivalent metabolites, methyl-arsonite ($MAs^{3+}$) and dimethylarsinite ($DMAs^{3+}$), inhibit insulin signaling in differentiated adipocytes, resulting in insulin resistance and impaired glucose uptake (Paul et al. 2007; Walton et al. 2004; Xue et al. 2011). The same arsenic species have also been shown to inhibit glucose-stimulated insulin secretion in isolated pancreatic islets or p cells (Douillet et al. 2013; Fu et al. 2010; Diaz-Villasenor et al. 2006, 2008). Consistent with these findings were results of population studies that found iAs exposure to be associated with insulin resistance (impaired insulin sensitivity) or β-cell dysfunction (Maull et al. 2012; Park et al. 2016). Additional mechanisms underlying the diabetogenic effects of iAs exposure may include: (i) inhibition of the differentiation of preadipocytes or myoblasts into adipocytes or myotubes (Wauson et al. 2002; Wang et al. 2005; Yen et al. 2010; Steffens et al. 2010), which could affect the capacity of adipose tissue and skeletal muscle to metabolize glucose, (ii) activation of gluconeogenesis in the fasting state (Liu et al. 2014; Huang et al. 2015), and (iii) altered methylation and/ or expression of genes associated with glucose metabolism and diabetes (Bailey et al. 2013; Diaz-Villasenor et al. 2007). Thus, multiple mechanisms may contribute to the development of diabetes associated with chronic exposure to iAs.

Surprisingly, little attention has been paid to the liver as a possible target in iAs-associated diabetes. Liver is a major glucose metabolizing organ involved in the regulation of glucose homeostasis (Klover and Mooney 2004), and also the organ that plays a central role in the metabolism of iAs (Drobna et al. 2009). The liver converts glucose into glycogen in fed state, while the breakdown of glycogen into glucose is critical for maintaining glucose homeostasis during fasting (Adeva-Andany et al. 2016). The metabolism of glucose in the liver is regulated by complex allosteric and hormonal mechanisms. Disruption of these mechanisms leads to impaired glucose homeostasis, which is often manifested by hyperglycemia and/or impaired glucose tolerance (Chung et al. 2014; Krssak et al. 2004; Lin and Accili 2011). Therefore, targeting hepatic glucose metabolism has become an important clinical practice in the treatment of type 2 diabetes (Rines et al. 2016).

The pathways of glucose metabolism in the liver include glycogenesis, glycogenolysis and gluconeogenesis (GNG). Glycogenesis is the pathway for glycogen synthesis during the fed state and glycogen synthase (GS) is the rate-limiting enzyme in this pathway (Dashty 2013). Both glycogenolysis and GNG are responsible for maintaining blood glucose levels in the fasting state. Glycogen phosphorylase (GP) is the rate-limiting enzyme in glycogenolysis, which breaks down glycogen to glucose (Agius 2015), while GNG synthesizes glucose from non-carbohydrate substrates (Sharabi et al. 2015). Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme in GNG. Insulin and glucagon regulate the rate-

limiting enzymes in all these pathways through transcriptional mechanisms and/or by covalent modification, i.e., phosphorylation and dephosphorylation (Rognstad 1979; Rosella et al. 1993). Only few studies have examined effects of iAs on these pathways and regulatory mechanisms. These studies found that chronic exposures to iAs may increase fasting glycemia by stimulating GNG through upregulation of PEPCK expression (Liu et al. 2014; Tseng 2004). In addition, acute exposures to toxic doses of iAs have been shown to stimulate glycogen depletion (Reichl et al. 1988; Verma et al. 2004). However, little data are available on the effects of low, environmentally relevant exposures to iAs on the pathways of glycogen metabolism and on mechanisms underlying these effects. Similarly, no data are available on effects on these pathways and mechanisms of the toxic methylated metabolites of iAs that are produced in the course of iAs metabolism in the liver (Thomas et al. 2007).

The goal of the present study was to characterize effects of iAs and its toxic methylated metabolite, $MAs^{3+}$, on glucose and glycogen metabolism in primary hepatocytes from mouse liver. Our results show that exposures to sub-cytotoxic concentrations of $iAs^{3+}$ and $MAs^{3+}$ decrease glycogen content in insulin-stimulated hepatocytes and that inhibition of GS and activation of GP, possibly due to insulin resistance, are the underlying mechanisms. These results outline a new mechanism by which arsenic exposure can disrupt glucose homeostasis, thus increasing risk of diabetes.

## Materials and methods

### Antibodies and other reagents

Primary antibodies were from Cell Signaling Technology (Danvers, MA, USA) unless otherwise noted. Secondary antibodies and SuperSignal West Pico chemiluminescent substrate were from Thermo Scientific (Waltham, MA, USA). Human recombinant insulin, sodium-D-lactate, sodium pyruvate, Avertin (2–2-2-tribromoethanol), phosphatase inhibitor cocktails 1 and 2, and Percoll were from Sigma-Aldrich (St. Louis, MO, USA). Protease inhibitor tablets were from Roche (Indianapolis, IN, USA). Type I collagenase was from Worthington Biochemical Corporation (Lakewood, NJ, USA). Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA, USA). UDP-[$^{14}$C] glucose was from American Radiolabeled Chemicals, Inc. (St. Luis, MO, USA).

### Hepatocyte isolation, culture and treatment with arsenicals

Hepatocytes were isolated from livers of anesthetized 8–15-week-old C57BL/6J mice by collagenase perfusion (Zhang et al. 2012, 2014) and seeded in 6-well or 12-well culture plates at density of $5.0 \times 10^5$/well or $2.0 \times 10^5$/well, respectively. The cell monolayers were cultured overnight in William's medium E (WME) (Invitrogen Carlsbad, CA, USA) with 1% penicillin/streptomycin and 2 mM glutamine, and with or without 10% fetal bovine serum (Hyclone Laboratories, South Logan, UT, USA). The hepatocytes were then exposed for 4 h to $iAs^{3+}$ (sodium arsenite, > 99% pure from Sigma) or $MAs^{3+}$ (methylarsine oxide, 98% pure) provided by Dr. William Cullen, University of British Columbia, Vancouver, Canada. Additional treatments are detailed below.

### Glycogen and free glucose assay

For this assay, the hepatocytes were cultured in serum-free WME overnight and then exposed to $iAs^{3+}$ or $MAs^{3+}$ for 4 h, with or without addition of 100 nM insulin for the last 2 h. Glycogen content was measured using a Glycogen Assay Kit from BioVision Inc. (Milpitas, CA, USA), following the manufacturer's instructions. In this assay, glycogen is enzymatically hydrolyzed to glucose, which is then oxidized to form an intermediate that reduces a colorless probe to a colored product with absorbance at 450 nm. When the glycogen hydrolyzing enzymes are omitted from the reaction mixture, this assay measures free intracellular glucose.

### GS activity assay

Glycogen synthase activity was determined by measuring incorporation of the $^{14}C$-glucosyl moiety of UDP-[$^{14}C$]glucose into glycogen (Thomas et al. 1968; Hue et al. 1975; Nuttall and Gannon 1989). Hepatocytes ($5.0 \times 10^5$) were homogenized in 0.5 ml of a buffer containing 100 mM NaF, 20 mM EDTA, 0.5% glycogen, 1% protease inhibitor, 1% phosphatase inhibitor cocktail, and 50 mM glycylglycine (pH 7.4). The homogenate was centrifuged at 9000×*g* for 10 min. Twenty microliters of the supernate was mixed with 100 μl reaction buffer containing 0.25 mM UDP-[$^{14}C$]glucose (~0.01 μCi/μmol), 1% glycogen, 10 mM $Na_2SO_4$, and 60 mM glycylglycine (pH 7.4). The mixture was incubated at 25 °C for 10 min (the reaction velocity was constant for up to 20 min). An aliquot of the reaction mixture (75 μl) was spotted on a filter paper. The paper was let to dry and then extracted twice with cold 66% ethanol—20 min for the first and 10 min for the second extraction. The filter paper was then washed with acetone for 5 min and dried. The radioactivity associated with the filter paper was measured by TRICARB 1900 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA, USA).

### GP activity assay

Glycogen phosphorylase activity was measured by determining the level of free phosphate that is released during the reversed reaction, i.e., synthesis of glycogen from glucose-1-phosphate (Hue et al. 1975; Saheki et al. 1985; Stalmans and Hers 1975). Hepatocytes were lysed in a buffer containing 100 mM NaF, 20 mM EDTA, 0.5% glycogen, 1% protease inhibitor, 1% phosphatase inhibitor cocktail, and 50 mM glycylglycine (pH 7.4); supernate obtained after 9000×*g* centrifugation was used for the assay. The reaction mixture contained 100 μl of the supernate, 100 μl of a reaction incubation buffer (2% glycogen, 100 mM glucose-1-phosphate) and 1 mM caffeine (to measure GPa activity) or 2 mM AMP (to measure total GP activity). After a 20-min incubation at room temperature (the reaction velocity was constant for up to 30 min), the reaction was terminated by addition 50 μl of 20% SDS. The concentration of inorganic phosphate was measured in 20 μl aliquots of the reaction mixtures by a colorimetric assay using a molybdate reagent (Saheki et al. 1985) and Synergy HT plate reader (BioTek, Winooski, VT, USA).

### Serine/threonine phosphatase activity assay

Serine/threonine phosphatase activity was assayed using the RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit (Molecular Probes, Eugene, OR, USA) which

measures inorganic phosphate produced by dephosphorylation of a synthetic substrate (provided in the kit). This assay is designed to measure activities of various serine/threonine phosphatases. The specificity of the assay depends on the reaction mixture composition. We used the reaction mixture that is recommended by the manufacturer for protein phosphatase 1 activity assay, i.e., the mixture containing 2 mM dithiothreitol (DTT) and 200 μM $MnCl_2$. The 9000×*g* supernate was prepared from hepatocytes as described above; 50 μl of the supernate was added into the reaction mixture and incubated in the dark for 20 min at room temperature (the reaction velocity was constant for up to 40 min). Fluorescence was measured at 358/452 nm using Synergy HT plate reader.

### Cell viability assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Styblo et al. 2000) was used to monitor viability of hepatocytes exposed for 4 h to $iAs^{3+}$ or $MAs^{3+}$ in the presence of insulin during the last 2 h of the exposure.

### Immunoblot analysis

Hepatocytes were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 mM $Na_3VO_4$, 25 mM NaF, 25 mM glycerophosphate, 2 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.3% Triton X-100, 1% protease inhibitor mix, and 1% phosphatase inhibitor cocktail. The lysates were separated by SDS-PAGE and transferred on PVDF membrane by electroblotting. Immunoblotting was performed to quantify target proteins. Horseradish peroxidase-conjugated secondary antibodies (ThermoFisher) were detected with SuperSignal West Pico chemiluminescent substrate by exposure to X-ray film. The film images were digitalized using an Epson scanner (Perfection 2400) and quantified using program Image J image processing program (NIH).

### Statistical analysis

A minimum of three biological replicates (i.e., hepatocytes from at least three mice) were used for each assay or immunoblot analysis, with two or three technical replicates each. Results from the technical replicates were averaged to obtain a representative value for each biological replicate. The values in figures are expressed as mean and SD for the biological replicates. Two types of statistical analyses were carried out. Differences between control (unexposed) cells and control cells treated with insulin were assessed by unpaired one-tail Student's *t* test (as the effects of insulin can be predicted using published data). Differences between insulin-treated control cells and insulin-treated cells exposed to different concentrations of $iAs^{3+}$ or $MAs^{3+}$ were evaluated by one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. For both types of analyses, differences with $p < 0.05$ were considered statistically significant and were marked in the figures describing experimental results; *p* values for marginally significant differences were also marked where appropriate.

## Results

### Exposure to $iAs^{3+}$ and $MAs^{3+}$ decreased glycogen and increased free glucose content in hepatocytes treated with insulin

We examined glycogen content in control hepatocytes cultured in basal WME medium and in hepatocytes exposed to $iAs^{3+}$ or $MAs^{3+}$ for 4 h, with or without addition of 100 nM insulin for the last 2 h of the exposure. In control hepatocytes that were not exposed to arsenicals, treatment with insulin resulted in an increase in glycogen content, while free glucose content decreased (Fig. 1a-d). Treatment with either $iAs^{3+}$ (0.5, 1, and 2 μM) or $MAs^{3+}$ (0.2, 0.5 and 1 μM) decreased glycogen content in the insulin-stimulated hepatocytes, thus offsetting the effect of insulin (Fig. 1a, b). Notably, exposure to 1 μM $MAs^{3+}$ decreased glycogen content by 57% as compared to a 43% decrease in hepatocytes exposed to 2 μM $iAs^{3+}$. While decreasing glycogen content, both $iAs^{3+}$ (1 and 2 μM) and $MAs^{3+}$ (1 μM) increased free glucose levels in insulin-stimulated hepatocytes. At these concentrations, neither $iAs^{3+}$ nor $MAs^{3+}$ affected cell viability measured by MTT assay (Fig. 1e, f). Cell viability was impaired only in hepatocytes exposed to 10 or 50 μM $iAs^{3+}$, the concentrations that were much higher than those used for assessment of the effects of $iAs^{3+}$ or $MAs^{3+}$ on glycogen content and on the glycogen metabolizing enzymes in this and the following experiments.

### Exposure to $iAs^{3+}$ and $MAs^{3+}$ did not induce PEPCK expression in hepatocytes treated with insulin

It has been previously reported that chronic exposure to $iAs^{3+}$ stimulates glucose production in GNG by inducing PEPCK expression (Liu et al. 2014; Tseng 2004). Thus, activation of GNG could be responsible for, or could contribute to the increased free glucose levels in hepatocytes treated with arsenicals. To assess possible role of GNG, we measured PEPCK protein levels in hepatocytes exposed to 2 μM $iAs^{3+}$ or 1 μM $MAs^{3+}$ for 4 h, with or without addition of 100 nM insulin for the last 2 h of the exposure. In spite of only 2-h treatment, insulin suppressed PEPCK protein level by 20%; a similar decrease was found in insulin-treated hepatocytes exposed to $iAs^{3+}$ (Supplemental Fig. 1). Exposure to $MAs^{3+}$ decreased PEPCK protein level by 65%. These results suggest that the increase in free glucose in insulin-stimulated hepatocytes exposed to $iAs^{3+}$ or $MAs^{3+}$ was not due to stimulation of GNG and was likely associated with changes in glycogen metabolism.

### Exposure to $iAs^{3+}$ and $MAs^{3+}$ inhibited GS activity and stimulated GP activity in hepatocytes treated with insulin

To determine whether the decrease in glycogen content in hepatocytes exposed to $iAs^{3+}$ or $MAs^{3+}$ was due to inhibition of glycogen synthesis or activation of glycogenolysis, we measured activities of GS and GP, the enzymes that catalyze the rate-limiting reactions in these pathways. The results show that treatment with insulin increased GS activity; however, exposures to $iAs^{3+}$ and $MAs^{3+}$ reversed the effect of insulin, decreasing GS activity in insulin-stimulated hepatocytes (Fig. 2a, b). GS activity in hepatocytes exposed to 1 μM $iAs^{3+}$ and 1 μM $MAs^{3+}$ and treated with insulin decreased by 55 and 45%, respectively. To further explore these findings, we compared GS activities in lysates of insulin-treated control hepatocytes in a regular reaction mixture and in a mixture, to which $iAs^{3+}$ or $MAs^{3+}$ was

added at final concentrations up to 10 μM. No differences in GS activities were found (data not shown) suggesting that inhibition of GS in hepatocytes exposed to $iAs^{3+}$ or $MAs^{3+}$ in culture and treated with insulin was not due to a direct interaction of the arsenicals with the enzyme, but rather due to effects on insulin-dependent mechanisms that regulate GS activity.

The GP assay used in this study measured activities of GPa (the activated, phosphorylated form of GP) and the total GP activity, i.e., sum of the activities of GPa and GPb (the less active-dephosphorylated form). Here, treatment with insulin, a negative regulator of GP (Aiston et al. 2003), decreased the relative GP activity (i.e., the ratio of GPa/total GP), but this effect was not statistically significant (Fig. 2c, d). The relative GPa activity in insulin-treated hepatocytes exposed to 2 μM $iAs^{3+}$ was significantly higher (1.3-fold) than in control insulin-treated hepatocytes; the effects of lower concentrations of $iAs^{3+}$ were not statistically significant. In comparison, exposure to $MAs^{3+}$ increased GP activity in insulin-treated cells at concentrations as low as 0.2 μM (1.7-fold) and 0.5 μM (2.2.-fold).

Further experiments carried out in this study focused on GS, which was significantly affected by exposure to both $iAs^{3+}$ and $MAs^{3+}$.

## Exposure to $iAs^{3+}$ and $MAs^{3+}$ inhibited GS dephosphorylaton in hepatocytes treated with insulin

The activity of GS in hepatocytes is regulated by phosphorylation. Specifically, phosphorylation on Ser-641 by glycogen synthase kinase 3 (GSK3) results in deactivation of GS and decreases glycogen synthesis (Rylatt et al. 1980; Imazu et al. 1984a, b). To determine whether altered GS phosphorylation was responsible for the decrease in GS activity in hepatocytes exposed to $iAs^{3+}$ or $MAs^{3+}$, we examined GS/Ser-641 phosphorylation status using immunoblot. As expected, insulin treatment decreased Ser-641 phosphorylation (Fig. 3). However, exposure to $iAs^{3+}$ and $MAs^{3+}$ reversed the effect of insulin, increasing Ser-641 phosphorylation by 2- and 2.2-fold in hepatocytes exposed to 2 μM $iAs^{3}$+ and 1 μM $MAs^{3}$+, respectively.

## Exposure to $iAs^{3+}$ and $MAs^{3+}$ inhibited insulin-dependent phosphorylation of protein kinase B, but had no effects on GSK3 phosphorylation

Glycogen synthase kinase 3 that phosphorylates GS on Ser-641 is regulated by insulin via phosphoinositide 3-kinase (PI3K)-protein kinase B (PKB/Akt) signaling pathway (Welsh et al. 1996). The phosphorylation of GSK3α on Ser-21 by phosphorylated PKB/Akt that results in downregulation of GSK3 kinase activity (Srivastava and Pandey 1998) is the mechanism by which insulin prevents phosphorylation deactivation of GS and inhibition of glycogen synthesis (Cross et al. 1995). We examined PKB/Akt and GSK3 phosphorylation status in hepatocytes exposed to $iAs^{3+}$ and $MAs^{3+}$ using immunoblot. We found that the insulin-dependent phosphorylation of Akt at Ser-473 and Thr-308, the two sites responsible for full activation of Akt (Alessi et al. 1996), is severely inhibited by exposure to either $iAs^{3+}$ or $MAs^{3+}$ (Fig. 4a-d). As expected, insulin treatment increased the phosphorylation of GSK3α/Ser-21; however, exposures to $iAs^{3+}$ and $MAs^{3+}$ had no effects on GSK3α/Ser-21 phosphorylation in hepatocytes treated with insulin (Fig. 4e-h).

### Exposure to $iAs^{3+}$ and $MAs^{3+}$ did not change serine/threonine phosphatase activity in hepatocytes

Another mechanism by which insulin activates GS involves activation of protein phosphatase 1 (PP1). PP1 is a serine/ threonine phosphatase that dephosphorylates (activates) GS (DePaoli-Roach et al. 2003; Roach et al. 1998). We examined serine/ threonine phosphatase activity in lysates from hepatocytes treated with $iAs^{3+}$ and $MAs^{3+}$ using a commercial kit from Molecular Probes and a reaction mixture containing 2 mM DTT and 200 μM $MnCl_2$ which, according to the manufacturer, should select specifically for PP1 activity. Using this assay mixture, we found no difference between the serine/threonine phosphatase activities in control hepatocytes and in hepatocytes exposed to either $iAs^{3+}$ or $MAs^{3+}$ (Supplemental Fig. 2). Notably, treatment with insulin did not increase the phosphatase activity in control hepatocytes suggesting that, despite the manufacturer's claim, the assay may not be specific for PP1 and that other, insulin-independent serine/ threonine phosphatases may contribute to the activity measured by this assay. However, because DTT chelates trivalent arsenic (Spuches et al. 2005), and thus may restore phosphatase activity in hepatocyte lysates after exposure to $iAs^{3+}$ or $MAs^{3+}$, we repeated the assay using the reaction mixture in which DTT was replaced with a non-thiol reductant tris(2-carboxyethyl) phosphine (TCEP). Notably, even with TCEP-containing reaction mixture no effects of $iAs^{3+}$ or $MAs^{3+}$ on the phosphatase activity were found (Supplemental Fig. 2).

## Discussion

Hepatic glycogenolysis and gluconeogenesis are the main sources of glucose for energy production between meals and during fasting. Dysregulation of glycogen metabolism may affect the energy-dependent metabolic and physiological processes in the body, and may also contribute to impaired glucose homeostasis as one of the attributes of diabetes (Krssak et al. 2004). Most of the previously published studies of the effects of iAs exposure on glycogen metabolism used acute treatments with high doses of iAs. For example, a significant decrease in hepatic glycogen content was reported in livers of mice and rats that were given a single or multiple doses of sodium arsenite or $As_2O_3$ (3–15 mg/kg b.w.) orally or by injection (Reichl et al. 1988; Verma et al. 2004; Berry and Smythe 1959; Reichl et al. 1991; Kawaguchi 1981; Albores et al. 1996; Singh et al. 2017; Huang et al. 2015). Similarly, repeated injections of $As_2O_3$, 2.5 mg/kg b.w. per day for five consecutive days, decreased glycogen content in livers of guinea pigs (Reichl et al. 1988). Only one study has examined effects of chronic iAs exposure at environmentally relevant levels (Huang et al. 2015). This study found that exposure to $iAs^{3+}$ in drinking water, 0.05 or 0.5 mg As/L for 6 weeks, decreased hepatic glycogen content in mice.

The present study builds on these findings. Our goal was to identify mechanisms that underlie the effects of iAs exposure on hepatic glycogen by examining the enzymes and pathways regulating glycogen metabolism in primary hepatocytes exposed to $iAs^{3+}$ and to its toxic methylated metabolite, $MAs^{3+}$. We found that $iAs^{3+}$ exposure decreased glycogen content in insulin-stimulated hepatocytes, which is consistent with results of the previous in vivo studies in laboratory animals. In addition, we showed that exposure to $MAs^{3+}$ also

reduced glycogen content, and that the decline in glycogen levels was associated with increased intracellular concentration of free glucose. Neither $iAs^{3+}$ nor $MAs^{3+}$ induced expression of PEPCK, the rate-limiting enzyme in GNG pathway. Thus, the decrease in glycogen content and the increase in glucose content in hepatocytes exposed to by $iAs^{3+}$ and $MAs^{3+}$ were either due to impairment in insulin-dependent utilization of glucose for glycogen synthesis or due to stimulation of glycogen breakdown to produce free glucose. Further investigation revealed that $iAs^{3+}$ and $MAs^{3+}$ altered both pathways by inhibiting insulin-stimulated GS activity and by simultaneously increasing GP activity. Because GS activity was inhibited by low concentrations of both $iAs^{3+}$ and $MAs^{3+}$ while only $MAs^{3+}$ was a potent activator of GP, our further investigation focused on GS as the common target for both arsenicals.

The hepatic GS is regulated by several protein kinases: AMP-activated kinase (AMPK) phosphorylates GS on Ser-7, casein kinase 2 (CK2) on Ser-10, and GSK3 on Ser-641, Ser-645, Ser-649 and Ser-653 (Ros et al. 2009; Imazu et al. 1984b; Rylatt et al. 1980). Mutagenesis studies have shown that phosphorylation on Ser-7, Ser-10, Ser-641, Ser-645, Ser-649 and Ser-653 plays a key role in GS regulation (Ros et al. 2009). GSK3 is negatively regulated by insulin (Welsh et al. 1996) suggesting that inhibition of insulin signaling resulting in GS phosphorylation by GSK3 and in downregulation of GS activity could be responsible for the decrease in glycogen content in hepatocytes exposed $iAs^{3+}$ and $MAs^{3+}$. To test this hypothesis, we examined the phosphorylation of GS on Ser-641, one of the targets of GSK3 kinase activity. As expected, insulin suppressed the phosphorylation of GS/Ser-641 in control hepatocytes, but exposures to $iAs^{3+}$ and $MAs^{3+}$ reversed the effect of insulin, resulting in a significant increase in Ser-641 phosphorylation. This result is consistent with inhibition of insulin signaling by $iAs^{3+}$ and $MAs^{3+}$, which has been previously reported in other studies, including studies carried out in our laboratory (Paul et al. 2007; Walton et al. 2004).

To further explore this mechanism, we examined effects of $iAs^{3+}$ and $MAs^{3+}$ on the insulin-activated signal transduction pathway, specifically on phosphorylation of PKB/Akt and of its downstream target, Ser-21 in GSK3α isoform. We found that treatment with insulin stimulated PKB/Akt phosphorylation in control hepatocytes and, in turn, GSK3α/Ser-21 phosphorylation also increased. The insulin-dependent phosphorylation of PKB/Akt was inhibited by both $iAs^{3+}$ and $MAs^{3+}$. This finding is consistent with results of our previous study in adipocytes that found both $iAs^{3+}$ and $MAs^{3+}$ to be potent inhibitors of phosphoinositide-dependent kinase-1 (PDK-1), which is responsible for PKB/Akt phosphorylation in the insulin signal transduction pathway (Paul et al. 2007). However, neither $iAs^{3+}$ nor $MAs^{3+}$ altered the insulin/PKB/Akt-dependent phosphorylation of GSK3α/ Ser-21. Notably, although the exposure to $iAs^{3+}$ and $MAs^{3+}$ inhibited insulin-dependent PKB/Akt phosphorylation, the levels of the phosphorylated PKB/Akt in the insulin-stimulated hepatocytes exposed to arsenicals were still higher than in control hepatocytes that were not treated with insulin (Fig. 4). Thus, it is possible that the residual PKB/Akt activity in arsenic-exposed and insulin-stimulated cells was sufficient to maintain GSK3α/Ser-21 phosphorylation at the level found in insulin-stimulated control cells. It is unclear, however, how the exposure to arsenical increased GS phosphorylation if GSK was phosphorylated, i.e., inactive.

Another mechanism by which insulin regulates GS activity involves activation of PP1 that dephosphorylates/ activates GS (Sharabi et al. 2015; Aiston et al. 2003). PP1 belongs to a larger family of protein phosphatases that also include PP2A, PP2B and PP2C (Cohen 1989). PP1 is a serine/threonine phosphatase with a relatively broad spectrum of substrates. The catalytic subunit of PP1 lacks substrate specificity. Affinity of PP1 towards specific substrates is conferred by a set of regulatory proteins that bind to the catalytic subunit of the enzyme (Peti et al. 2012). Thus, although GS is considered one of the primary targets of PP1, measuring the GS specific PP1 activity is difficult because various forms of PP1 with different substrate specificities are present in biological samples. The choice of methods/kits is very limited. In this study, we used a commercial 96 EnzChek® Serine/Threonine Phosphatase Assay Kit from Molecular Probes (R-33700). This kit uses 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as a substrate for several Ser/Thr phosphatase, including PP1, PP2A, PP2B and PP2C. DiFMUP is dephosphorylated to produce fluorescent 6,8-difluoro-4-methylumbelliferyl (DiFMU). The manufacturer recommends specific components for the assay mixture to measure activities of particular Ser/Thr phosphatases. We used the composition that, according to the manufacturer, selects for PP1 activity, using first DDT (recommended) and then TCEP as a reductant. However, the fact that we did not observe any increase in the phosphatase activity in control hepatocytes treated with insulin, the PP1 activator (Supplemental Fig. 2), strongly suggests that the assay conditions were not PP1 specific. Thus, the role of PP1 in the inhibition of GS activity in hepatocytes exposed to $iAs^{3+}$ and $MAs^{3+}$ remains unclear.

In summary, the present study provides additional evidence that exposure to iAs may result in a reduction or depletion of glycogen stores in the liver and suggests that $MAs^{3+}$, the intermediary metabolite formed in the pathway for iAs methylation, can significantly contribute to this outcome. This study is the first to show that exposure to $iAs^{3+}$ and $MAs^{3+}$ decreases glycogen content in hepatocytes by inhibiting the insulin-dependent phosphorylation (activation) of GS. $MAs^{3+}$ and, to a lesser extent $iAs^{3+}$, also stimulated GP activity. Thus, both GS inhibition and GP activation contribute to glycogen depletion, in particular in hepatocytes exposed to $MAs^{3+}$. While we were not able to determine the roles of GSK3 or PP1 in the inhibition of GS activity by $iAs^{3+}$ and $MAs^{3+}$, our results point to inhibition of insulin signaling (i.e., insulin resistance) as the key underlying mechanism. Future studies should validate these findings in laboratory animal models and should also examine potential roles of other kinases that phosphorylate hepatic GS, specifically AMPK and CK2.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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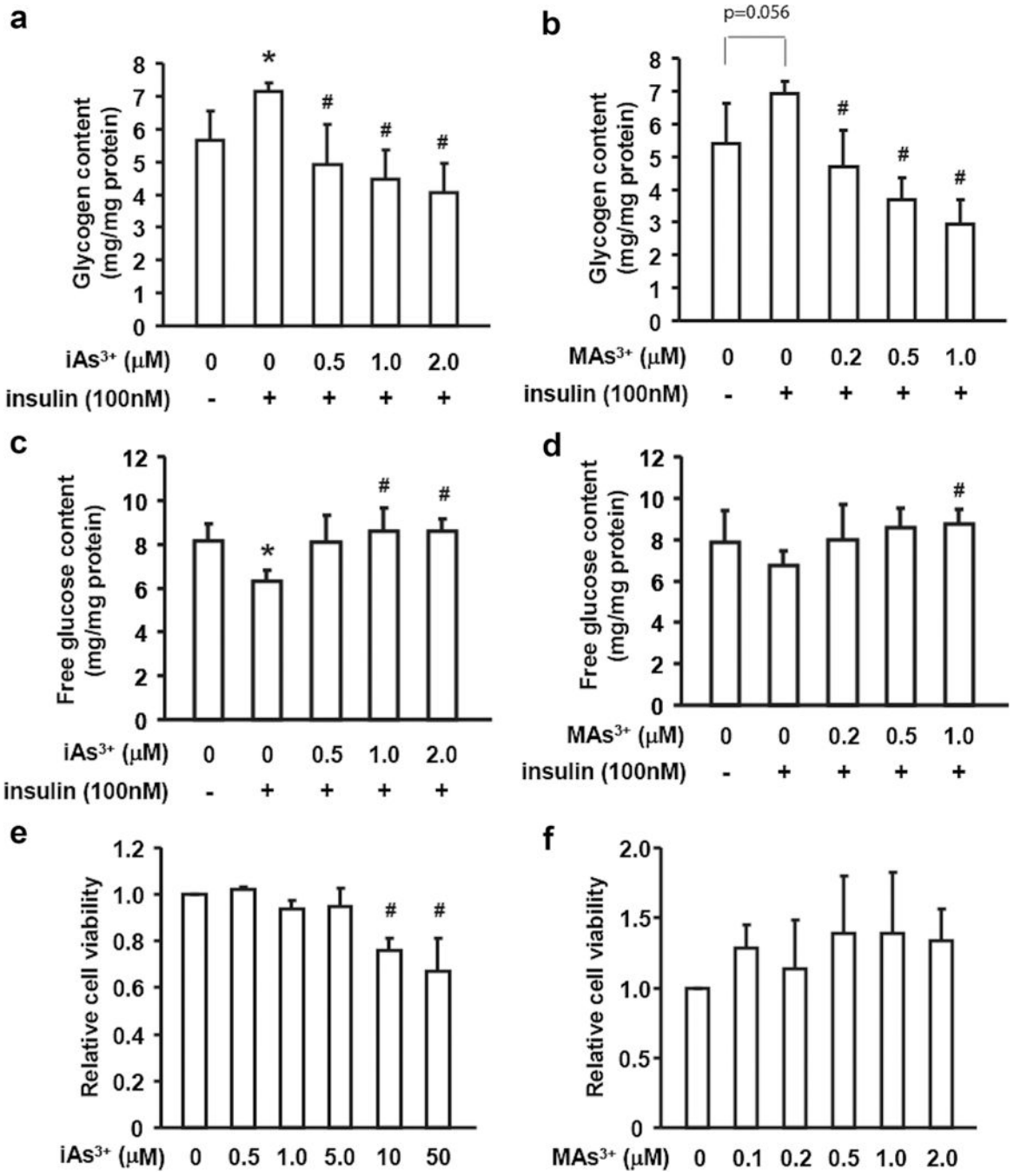


**Fig. 1.**

Exposures to iAs and $MAs^{3+}$ decreased glycogen content and increased free glucose concentration in primary murine hepatocytes. Hepatocytes were exposed to $iAs^{3+}$ (**a**, **c**) or $MAs^{3+}$ (**b**, **d**) for 4 h with or without insulin treatment (100 nM) for the last 2 h of the exposure. Glycogen (**a**, **b**) and free glucose (**c**, **d**) were measured in cell lysates. MTT assay was used to assess cell viability in hepatocytes exposed to $iAs^{3+}$ (**e**) or $MAs^{3+}$ (**f**) for 4 h with insulin treatment (100 nM) for the last 2 h of the exposure. Values are expressed as mean and SD for three independent experiments. *Statistically significant differences ($p <$

0.05) between control hepatocytes and control hepatocytes treated with insulin, and #between insulin-treated control hepatocytes and insulin-treated hepatocytes exposed to arsenicals; *p* values for marginally significant differences are also shown

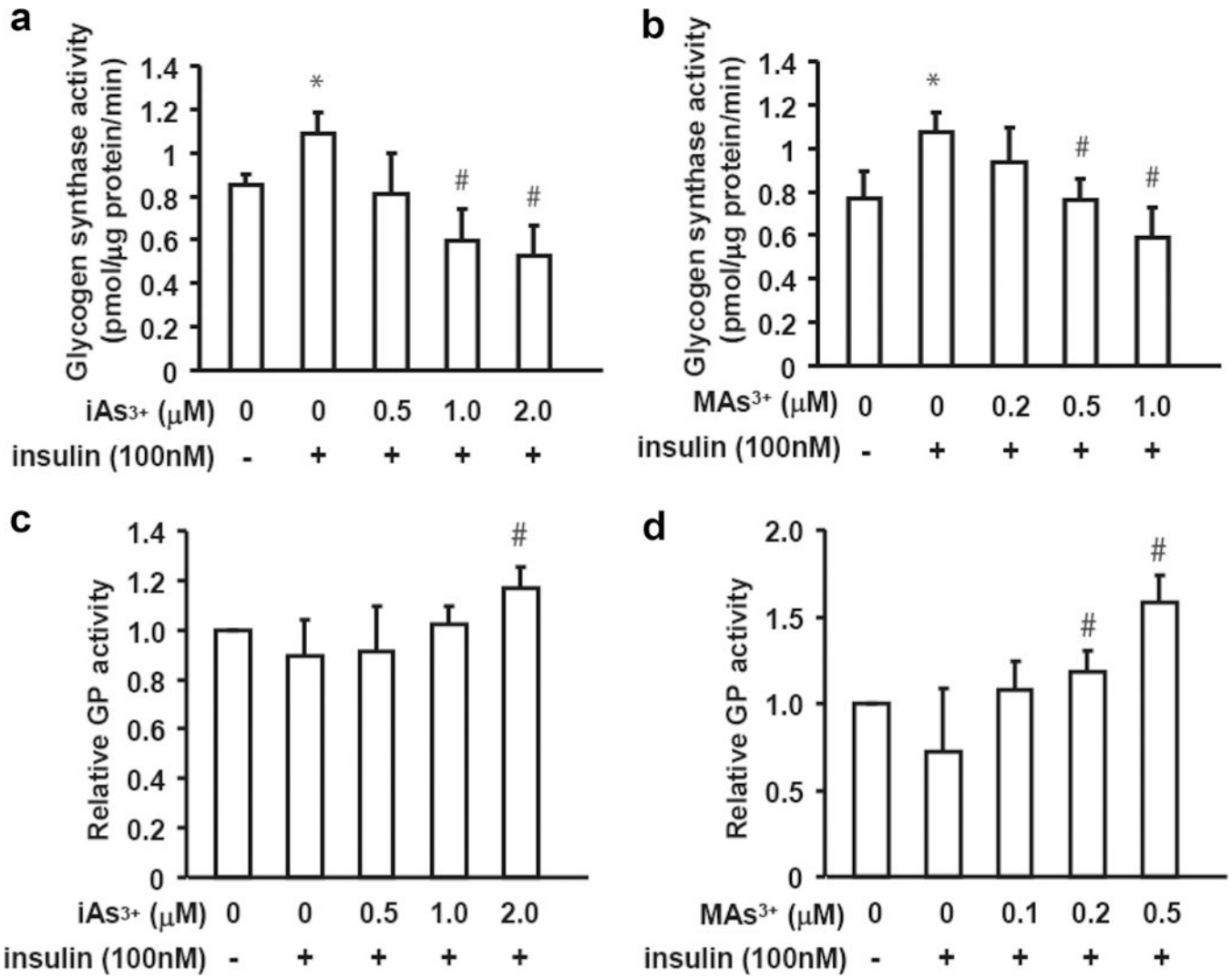


**Fig. 2.**

Exposures to iAs and $MAs^{3+}$ inhibited the insulin-dependent activation of glycogen synthase and activated glycogen phosphorylase. Hepatocytes were exposed to $iAs^{3+}$ (**a**, **c**) or $MAs^{3+}$ (**b**, **d**) for 4 h with or without insulin treatment (100 nM) for the last 2 h of the exposure. Glycogen synthase (**a**, **b**) activity and the relative glycogen phosphorylase activity (i.e., GPa activity/total GP activity) (**c**, **d**) were measured in cell lysates. Values are expressed as mean and SD for three independent experiments. *Statistically significant differences ($p < 0.05$) between control hepatocytes and control hepatocytes treated with insulin, and #between insulin-treated control hepatocytes and insulin-treated hepatocytes exposed to arsenicals

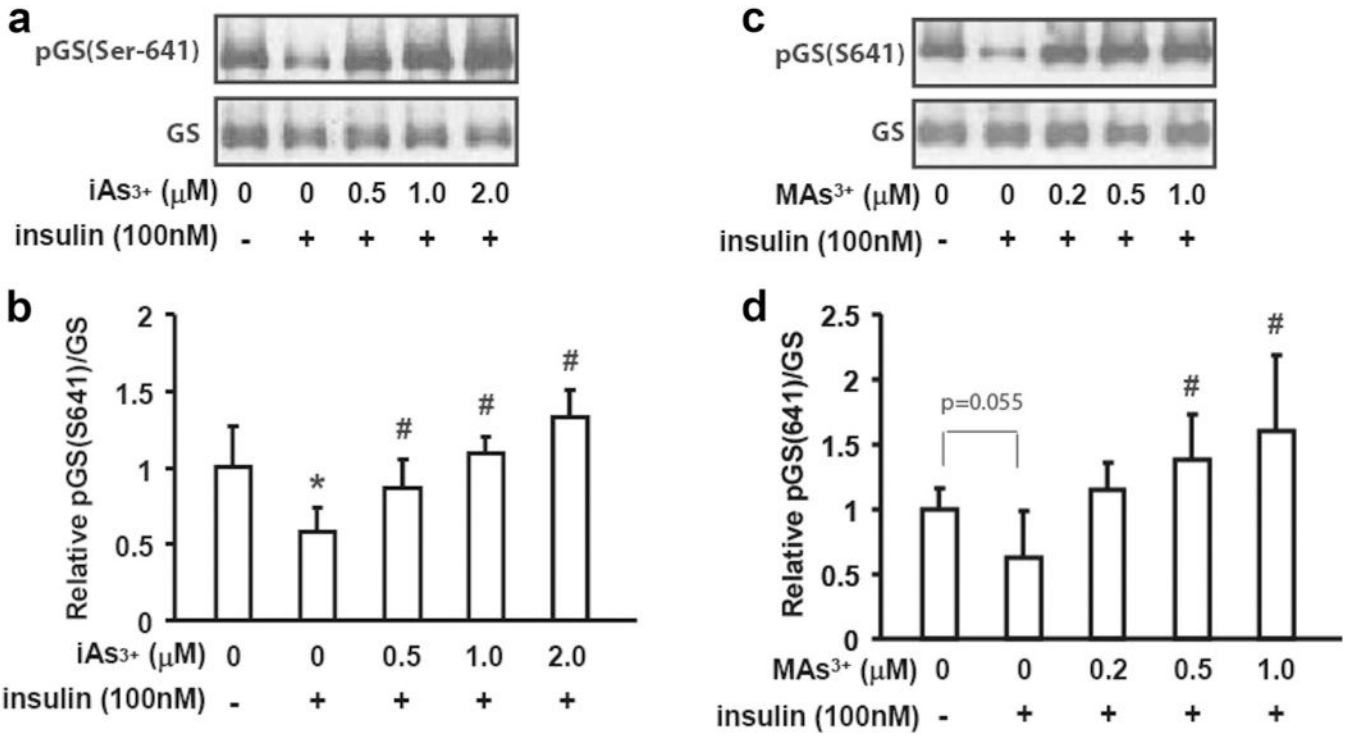


**Fig. 3.**

Exposures to $iAs^{3+}$ and $MAs^{3+}$ inhibited the insulin-dependent dephosphorylation of glycogen synthase. Hepatocytes were exposed to $iAs^{3+}$ (**a**, **b**) or $MAs^{3+}$ (**c**, **d**) for 4 h with or without insulin treatment (100 nM) for the last 15 min of the exposure. The total glycogen synthase (GS) levels and the phosphorylation of glycogen synthase on Ser-641 [pGS(Ser-641)] were assessed by immuno- blot (**a**, **c**). The immunoblot data were quantified using the ratio of pGS(Ser-641)/GS (**b**, **d**). Representative data from four independent experiments are shown; values in b and d are expressed as mean and SD. *Statistically significant differences ($p < 0.05$) between control hepatocytes and control hepatocytes treated with insulin, and #between insulin-treated control hepatocytes and insulin-treated hepatocytes exposed to arsenicals; $p$ values for marginally significant differences are also shown

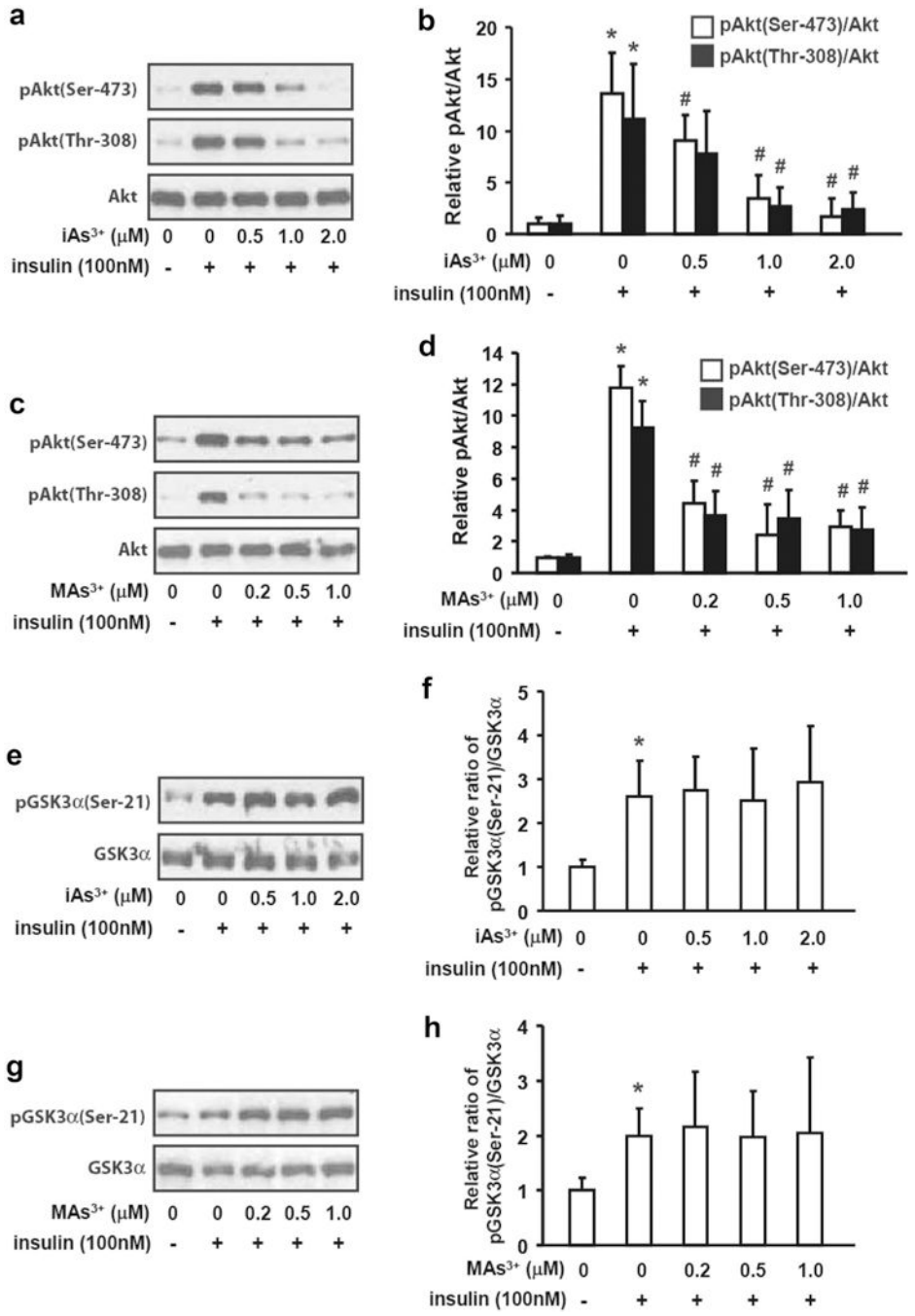


**Fig. 4.**

Exposures to iAs $^{+}$ and MAs$^{3}$+ inhibited the insulindependent phosphorylation of PKB/Akt bud had no effects on GSK3 phosphorylation. Hepatocytes were exposed to iAs$^{3+}$ (**a**, **b**, **e**, **f**) or MAs$^{3+}$ (**c**, **d**, **g**, **h**) for 4 h with or without insulin treatment (100 nM) for the last 15 min of the exposure. The total Akt and phospho-Akt [pAkt(Ser-473) and pAkt(Thr-308)] levels (a, c), and total GSK3α and phosphor- GSK3α [pGSKa(Ser-21)] (**e**, **g)** were assessed by immunoblot. The immunoblot data were quantified using the ratios of pAkt/Akt (**b**, **d**) and pGSK3α(Ser-21)/GSK3α (**f**, **h**). Representative data from three independent experiments

are shown; values in **b**, **d**, **f** and **h** are expressed as mean and SD. *Statistically significant differences ($p < 0.05$) between control hepatocytes and control hepatocytes treated with insulin, and #between insulin-treated control hepatocytes and insulin-treated hepatocytes exposed to arsenicals