Prolonged inorganic arsenite exposure suppresses insulin-stimulated AKT S473 phosphorylation and glucose uptake in 3T3-L1 adipocytes: Involvement of the adaptive antioxidant response

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Abstract

There is growing evidence that chronic exposure of humans to inorganic arsenic, a potent environmental oxidative stressor, is associated with the incidence of type 2 diabetes (T2D). One critical feature of T2D is insulin resistance in peripheral tissues, especially in mature adipocytes, the hallmark of which is decreased insulin-stimulated glucose uptake (ISGU). Despite the deleterious effects of reactive oxygen species (ROS), they have been recognized as a second messenger serving an intracellular signaling role for insulin action. Nuclear factor erythroid 2-related factor 2 (NRF2) is a central transcription factor regulating cellular adaptive response to oxidative stress. This study proposes that in response to arsenic exposure, the NRF2-mediated adaptive induction of endogenous antioxidant enzymes blunts insulin-stimulated ROS signaling and thus impairs ISGU. Exposure of differentiated 3T3-L1 cells to low-level (up to 2 μM) inorganic arsenite (iAs^{3+}) led to decreased ISGU in a dose- and time-dependent manner. Concomitant to the impairment of ISGU, iAs^{3+} exposure significantly attenuated insulin-stimulated intracellular ROS accumulation and AKT S473 phosphorylation, which could be attributed to the activation of NRF2 and induction of a battery of endogenous antioxidant enzymes. In addition, prolonged iAs^{3+} exposure of 3T3-L1 adipocytes resulted in significant induction of inflammatory response genes and decreased expression of adipogenic genes and glucose transporter type 4 (GLUT4), suggesting chronic inflammation and reduction in GLUT4 expression may also be involved in arsenic-induced insulin resistance in adipocytes. Taken together our studies suggest that prolonged low-level iAs^{3+} exposure activates the cellular adaptive oxidative stress response, which impairs insulin-stimulated ROS signaling that is involved in ISGU, and thus causes insulin resistance in adipocytes.

Keywords

arsenic; diabetes; insulin; ROS; NRF2; oxidative stress

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Introduction

There is growing evidence that environmental inorganic arsenic (iAs) exposure is associated with the incidence of Type 2 diabetes (T2D), a metabolic disease attributed to a combination of genetic and environmental factors. In T2D patients, insulin resistance in target tissues, including the adipose tissue, muscle and liver, is the central pathophysiological event in the development of the disease. While a number of mechanisms, including increased levels of free fatty acids, inflammatory cytokines and adipokines, hyperinsulinemia and mitochondrial dysfunction, have been suggested to be responsible for insulin resistance [1], compelling evidence indicates that increased oxidative stress and inflammatory response play prominent roles [2; 3; 4; 5]. Accumulating data, including our own studies *in vitro* [6; 7], *in vivo* [8], and in humans [9], reveal that iAs exposure results in oxidative stress, suggesting that oxidative stress may be involved in the development of insulin resistance in humans exposed to iAs.

A key cellular component that defends cells against oxidative toxicity is the Nuclear factor erythroid 2 related factor 2 (NRF2), a transcription factor that is directly responsible for both basal and inducible expression of many antioxidant/detoxification enzymes [10]. The NRF2-mediated antioxidant induction has been recognized as one of the most important cellular adaptive responses to oxidative stress. However, this NRF2-driven induction of endogenous antioxidant enzymes - meant to maintain intracellular redox homeostasis and limit oxidative damage - may also have an adverse effect, i.e., to block the reactive oxygen species (ROS) that function as physiological signaling molecules [11; 12]. In adipocytes, ROS, in particular hydrogen peroxide (H_2O_2), are involved in insulin signal transduction leading to phosphorylation of protein kinase B (PKB/AKT) and glucose uptake [13]. Thus, persistent activation of NRF2 in response to chronic exposure to iAs and/or its trivalent metabolites may blunt insulin-triggered H_2O_2 signaling, resulting in reduced insulin sensitivity in adipocytes. Here we report that prolonged exposure to low-levels of inorganic arsenite (iAs^{3+}) activates NRF2-mediated antioxidant response, impairs insulin signaling, such as phosphorylation of AKT S473, and suppresses insulin-stimulated glucose uptake (ISGU).

Materials and Methods

Chemicals

Sodium arsenite, dexamethasone, 3-isobutylmethylxanthine, insulin and Oil Red O (ORO) were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) with high glucose, fetal bovine serum (FBS), 10 % bovine serum albumin (BSA), phosphate buffered saline (PBS), calf serum, TRIzol and blue-fluorescent DAPI nucleic acid stain were purchased from Invitrogen (Carlsbad, CA). [^{3}H]-2-deoxy-D-glucose ([^{3}H]-2-DG) was obtained from PerkinElmer, Inc. (Waltham, MA).

Cell culture, differentiation and treatments

3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA) and maintained in high-glucose DMEM with 50 unit/ml penicillin, 50 μg/ml streptomycin, and 10% calf serum. All the cells were maintained at 37 °C in a 5% CO_2 environment. Adipogenic differentiation was induced by using 1 μM dexamethasone, 0.5 mM 3-isobutylmethylxanthine and 5 μg/ml insulin in DMEM with 10% FBS (DMI protocol) as described previously [14]. Differentiation of preadipocytes to mature adipocytes was confirmed by ORO staining of lipid vesicles [14]. Fully-differentiated 3T3-L1 cells were exposed to low levels of iAs^{3+} (up to 2 μM) for 7 days followed by immediate sample collection and measurements. To determine insulin-stimulated phosphorylation of AKT S473, following 7-day iAs^{3+}

treatment, 3T3-L1 adipocytes were first starved for 4 hr in serum-free DMEM containing 1 % BSA while the same concentrations of iAs^{3+} were maintained in the media. The starved cells were then challenged with insulin (5 ng/ml in DMEM) for 5 min, and followed by immediate cell lysate isolation.

Glucose uptake assay

Glucose uptake in 3T3-L1 adipocytes was measured as described previously [15]. Briefly, after 4- or 7-day treatment with iAs^{3+}, 3T3-L1 adipocytes were plated in a 12-well plate and starved for 4 hr as above. Glucose uptake was determined by the accumulation of $[^3H]$-2-DG in the cells by a 20 min incubation with 0.5 μCi $[^3H]$-2-DG in 1 ml glucose-free DMEM with 50 ng/ml insulin or vehicle (medium). The radioactivity of cell lysates was determined in 5 ml of Ecolume (MP Biomedicals, LLC, Solon, OH) using Tri-carb Liquid Scintillation Analyzer (Packard Instrument Co. Inc. Meriden, CT) and normalized with protein level. ISGU was then determined as follows after non-specific binding of $[^3H]$-2-DG with cell surface was subtracted: (Radioactivity with insulin - Radioactivity with Vehicle)/ Radioactivity with Vehicle.

Quantitative real-time RT–PCR analysis

Total RNA was isolated with TRIzol. Quantitative real-time RT-PCR was performed as described previously [16]. The primers (sequences are shown in Table S1, Supplemental Materials) were designed using Primer Express 4 (Applied Biosystems, Foster City, CA) and synthesized by MWG-BIOTECH Inc. (High Point, NC). Real-time fluorescence detection was carried out using an ABI PRISM 7900 HT Sequence Detector (Applied Biosystems).

Western blot analysis

Isolation of cell fractions and Western blotting were performed as described previously [6]. Antibodies for NRF2 (sc-13032; 1: 500), heme oxygenase 1 (HO-1, sc-136902; 1: 500) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody for NAD(P)H: quinone oxidoreductase 1 (NQO1, 39-3700; 1: 1000) was from Invitrogen. Antibody for phosphorylated AKT S473 (p-AKT S473, #9271; 1: 500) and total AKT (t-AKT, #4685; 1: 500) was from Cell Signaling Technology, Inc. (Danvers, MA). Antibody for β-ACTIN (A1978; 1: 2000) was from Sigma.

Determination of total intracellular glutathione (GSH)

Cells were sonicated in cold PBS immediately after collection followed by centrifugation at $12,000 \times g$ for 5 min. The resulting supernatants were used for measurement of total intracellular glutathione using the BIOXYTECH GSH/GSSG-412 kit (OxisResearch, Portland, OR) [17].

Intracellular peroxide determination

Intracellular peroxide levels in 3T3-L1 adipocytes were measured by fluorescence microscopy using the fluorescent probe 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen) as described previously [18]. The final concentration of CM-H_2DCFDA used was 2 μM and loading time was 30 min. The fluorescence images were obtained by using an Axio Observer Z1 fluorescence microscope (Carl Zeiss, Inc., Jena, Germany) and quantified by using ImageJ 1.44 (http://rsbweb.nih.gov/ij/).

Immunofluorescence staining

Cells were grown on glass coverslips in 6-well plate. After 7-day treatment with iAs^{3+}, cells were fixed with 2 % formaldehyde overnight and permeabilized with 0.3 % Triton X-100 in PBS for 10 min at room temperature. After blocking in 10 % serum in PBS for 1 hr, cells were then incubated with anti-glucose transporter type 4 (GLUT4, sc-7938; 1: 50) antibody overnight at 4 °C. Coverslips were washed and incubated with Goat anti-rabbit IgG-FITC secondary antibody (sc-2012; 1:50). After DAPI staining, the coverslips were mounted on glass slides and examined using an Axio Observer Z1 fluorescence microscope.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 4 (GraphPad Software, San Diego, CA), with $p < 0.05$ taken as significant. Data are expressed as mean ± SEM. For comparisons among groups, one-way or two-way ANOVA with Bonferroni post hoc testing was performed.

Results

Prolonged iAs^{3+} exposure suppressed insulin-stimulated phosphorylation of AKT S473 and glucose uptake in 3T3-L1 adipocytes

3T3-L1 cells, derived from mouse embryo fibroblasts, have been used as a model of adipogenic differentiation and insulin action. Upon hormonal stimulation, the cells undergo growth arrest and initiate differentiation manifested by the morphological and enzymatic characteristics of mature adipocytes, such as large lipid droplet (Supplemental Materials Fig. S1). During differentiation, 3T3-L1 cells increase the expression of insulin receptors and their capacity to bind insulin ligand [19]. Thus, 3T3-L1 adipocytes are a well accepted cell model to study insulin signaling.

The effect of chronic iAs^{3+} exposure on insulin sensitivity was investigated by measuring phosphorylation of AKT S473 and glucose uptake in 3T3-L1 adipocytes challenged with insulin following a 7-day iAs^{3+} exposure at non-cytotoxic concentrations (Fig. S1). As shown in Fig. 1A, acute insulin stimulation resulted in a dramatic increase in AKT S473 phosphorylation in Control cells. In contrast, insulin-stimulated AKT S473 phosphorylation was attenuated in iAs^{3+}-exposed cells despite their moderately elevated basal levels in the absence of insulin. In keeping with the attenuation of AKT phosphorylation, iAs^{3+} exposure led to reduced ISGU in a time- and dose-dependent fashion (Fig. 1B).

Prolonged iAs^{3+} exposure activated NRF2 and the antioxidant response and dampened insulin-stimulated ROS production in 3T3-L1 adipocytes

The effect of chronic iAs^{3+} exposure on NRF2-mediated antioxidant response was investigated by measuring the expression of NRF2 and its downstream target antioxidant genes in fully differentiated 3T3-L1 cells. As shown in Fig. 2A and B, prolonged exposure (7 days) of 3T3-L1 adipocytes to iAs^{3+} resulted in a dose-dependent increase in the expression of NRF2 at mRNA and protein levels. Consistent with these findings, significant induction of NRF2 target genes, including γ-glutamate cysteine ligase catalytic subunit (GCLC) and regulatory subunit (GCLM), glutathione *S*-transferase (GST), NQO1 and HO-1, was also observed in iAs^{3+}-treated cells (Fig. 2C and D, and Supplemental Materials Fig. S2). In addition, intracellular GSH levels showed a similar pattern to NRF2 and its target gene expression (Fig. 2E). Furthermore, the expression of many inflammatory factors, including tumor necrosis factor α (*Tnfα*), interleukin 6 (*Il6*), interleukin 1β (*Il1β*), cyclooxygenase 2 (*Cox2*) and inducible nitric oxide synthase (*iNos)*, was also significantly induced in response to prolonged iAs^{3+} exposure (Supplemental Materials Fig. S3). These

results clearly demonstrate prolonged, low-level iAs^{3+} exposure activates NRF2-mediated antioxidant response, as well as inflammatory response in 3T3-L1 adipocytes.

Intracellular peroxide levels were determined to investigate whether iAs^{3+} exposure affects insulin-stimulated ROS signaling in 3T3-L1 adipocytes. Consistent with the premise that ROS is a second messengers mediating insulin signaling [13], as shown in Fig. 3, insulin significantly increased the intracellular peroxide level in Control cells. As expected, cells exposed to iAs^{3+} alone, a potent oxidative stressor, exhibited high basal peroxide levels. However, when the iAs^{3+}-exposed cells were stimulated with insulin, no additional increase in the peroxide level was observed, suggesting that the elevated antioxidants after prolonged iAs^{3+} exposure (Fig. 2 and Supplemental Materials Fig. S2) may blunt the production of any signaling ROS that are stimulated by insulin. The abolished peroxide response is likely responsible, at least partially, for the mitigated phosphorylation of AKT S473 and ISGU observed in iAs^{3+}-exposed cells (Fig. 1).

Effect of prolonged iAs^{3+} exposure on the expression of adipogenic genes and GLUT4 in 3T3-L1 adipocytes

Peroxisome proliferator activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) are expressed abundantly in adipose tissue and is considered to be the dominant transcriptional regulator of adipogenic differentiation and adipocyte function [14]. As shown in Fig. 4A, prolonged exposure of 3T3-L1 adipocytes to low levels of iAs^{3+} resulted in markedly reduced gene expression of *Pparγ1*, *Pparγ2*, *Cebpα* and *Glut4*. In keeping with the reduced gene expression of *Glut4*, immunofluorescence microscopy also showed a substantial decrease in the GLUT4 protein level in iAs^{3+}-exposed 3T3-L1 adipocytes (Fig. 4B).

Discussion

While the precise mechanisms for the diabetogenic effect of iAs are still largely undefined, recent experimental studies indicated that acute exposure to iAs or its metabolites impairs ISGU and results in insulin resistance [15; 20; 21]. The present study reveals that prolonged low-level iAs^{3+} exposure of 3T3-L1 adipocytes causes oxidative stress and inflammatory response and impairs insulin-stimulated phosphorylation of AKT S473 and ISGU. The concurrence of iAs^{3+}-induced activation of NRF2-mediated antioxidant response, attenuation of insulin-stimulated peroxide production, AKT S473 phosphorylation and ISGU suggests that chronic iAs exposure-induced insulin resistance in adipocytes may result, at least partially, from activation of NRF2 and subsequent induction of antioxidant enzymes, which might dampen ROS-mediated signaling transduction by insulin.

Traditionally, ROS have been thought of as useless by-products of respiratory metabolism in mitochondria, and are generally believed to be deleterious to biological systems. In the field of T2D research, oxidative stress from excessive ROS production is recognized as a chief culprit in the development of insulin resistance in insulin-responsive cells [5]. Despite the deleterious effects of ROS, growing evidence indicates that they can function as a second messenger serving an intracellular signaling role [22]. With respect to T2D, it has been shown that ROS, in particular H_2O_2, is involved in insulin signal transduction in insulin-responsive cells [23]. In adipocytes, NADPH oxidase has been identified as an important source of ROS production in response to insulin stimulation [23]. Based on these studies it can be argued that augmentation of the cellular ROS-scavenging capacity, as a result of the adaptive response to oxidative stress, may actually impair the 'beneficial' aspects of ROS signaling that contribute to insulin action. Thus, we propose that NRF2-mediated adaptive antioxidant response plays paradoxical roles in insulin-responsive cells: (1) it protects these cells from oxidative damage; but (2) it also blunts insulin-triggered 'ROS signaling' and

thus results in reduced insulin sensitivity in insulin-responsive cells. Clearly, the second premise may be relevant to low-dose iAs-induced insulin resistance in adipocytes as oxidative damage may not be significant at these low doses.

Consistent with the idea that activation of NRF2 may be involved in the development of insulin resistance, elevated levels of antioxidants have been observed in *ob/ob* mice [24], subjects at risk of T2D [25] and diabetic patients [26; 27]. More importantly, emerging *in vivo* data suggest that overexpression of H_2O_2-scavenging enzymes, such as catalase and glutathione peroxidase 1, actually increased insulin resistance and the likelihood of developing diabetes despite potentially improved handling of oxidative stress [28; 29]. Together with our previous study [17] showing that low levels of iAs^{3+} impair glucose-stimulated insulin secretion in pancreatic β-cells, which is also associated with the adaptive cellular oxidative stress response, iAs-induced adaptive induction of antioxidants stress may be an important general mechanism for the development of T2D.

GLUT4 is an insulin-regulated glucose transporter highly expressed in the adipose tissue and skeletal muscle [30]. Transgenic mice expressing high levels of GLUT4 in the adipose tissue are insulin sensitive and glucose tolerant. In contrast, conditional depletion of GLUT4 in the adipose tissue causes insulin resistance [30]. In the absence of insulin, GLUT4 is sequestered in the lipid bilayers of intracellular vesicles of fat and muscle cells. Insulin induces the translocation of GLUT4 from the intracellular store to the plasma membrane and thus increases glucose uptake. Although insulin-stimulated GLUT4 translocation, a major downstream event of insulin-stimulated AKT activation, is one of the most important mechanisms in regulating ISGU, the observed downregulation of GLUT4 expression by iAs (Fig. 4) suggests that another mechanism may also be at work to contribute to iAs-induced insulin resistance in adipocytes. Obviously, the exact mode of GLUT4 regulation by iAs at the transcriptional or translational level needs further investigation.

Taken together, this study indicates that low concentrations of iAs cause oxidative stress and cellular adaptive response involving NRF2 activation, in adipocytes, and such a cellular adaptive response to iAs is associated with impairment of insulin sensitivity. While the etiology of T2D is still unclear, to date, therapeutic approaches have focused on medication and lifestyle modification. The role of environmental exposures should also be considered in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

BSA	bovine serum albumin
C/EBPα	CCAAT-enhancer-binding protein α

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CM-H$_2$DCFDA	5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester
COX2	cyclooxygenase 2
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
GCLC	γ-glutamate cysteine ligase catalytic subunit
GCLM	γ-glutamate cysteine ligase regulatory subunit
GSH	Glutathione
GST	glutathione S-transferase
[^{3}H]-2-DG	[^{3}H]-2-deoxy-D-lucose
HO-1	heme oxygenase 1
H$_2$O$_2$	hydrogen peroxide
iAs	inorganic arsenic
IL1β	interleukin 1β
IL6	interleukin 6
GLUT4	glucose transporter type 4
iAs^{3+}	inorganic arsenite
iNos	inducible nitric oxide synthase
ISGU	insulin-stimulated glucose uptake
NQO1	NAD(P)H: quinone oxidoreductase 1
NRF2	Nuclear factor erythroid 2-related factor 2
ORO	Oil Red O
PKB/AKT	protein kinase B
PBS	phosphate buffered saline
PPARγ	peroxisome proliferator activated receptor γ
ROS	reactive oxygen species
T2D	type 2 diabetes
TNFα	tumor necrosis factor α

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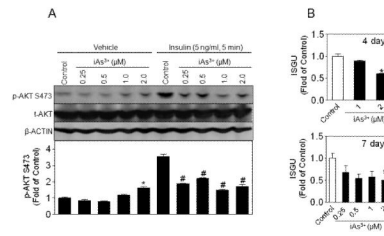
Fig 1.
Prolonged low-level iAs^{3+} exposure inhibits insulin-stimulated phosphorylation of AKT S473 and ISGU in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated using dexamethasone, 3-isobutylmethylxanthine and insulin for 7 days followed by a 7-day iAs^{3+} exposure. (A) Immunoboltting of p-AKT S473 and t-AKT in whole cell lysates, with β-ACTIN as a loading control. Lower panel is the quantitative result of p-AKT S473. n = 3; * $p < 0.05$ vs. Control with Vehicle (medium); # $p < 0.05$ vs. Control with Insulin. (B) ISGU measured in 3T3-L1 adipocytes that had been exposed to low-level iAs^{3+} for 4 days (upper panel) and 7 days (lower panel). n = 3; *$p < 0.05$ vs. Control.
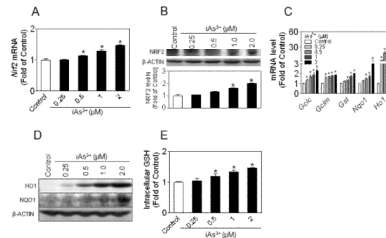
Fig. 2.
iAs^{3+} activates NRF2-mediated antioxidant response in 3T3-L1 adipocytes. 3T3-L1
adipocytes were exposed to iAs^{3+} for 7 days. (A) Gene expression of *Nrf2*. n = 3–6; *, $p <$
0.05 vs. Control. (B) Protein level of NRF2 in whole cell lysates. β-ACTIN is a loading
control. Lower panel is the quantified result of immunoblots. n = 3; * $p < 0.05$ vs. Control.
(*C*) Expression of NRF2-target genes measured by real-time RT-PCR. n = 3; * $p < 0.05$ vs.
Control. (D) Protein expression of NRF2 downstream target HO1 and NQO1. (*E*)
Intracellular GSH level. GSH levels in whole cell lysates were normalized by protein
content and expressed as fold of Control. The intracellular GSH concentration in Control
cells is 20.7 ± 2.2 μmol/g protein. n = 3; * $p < 0.05$ vs. Control.

A

B
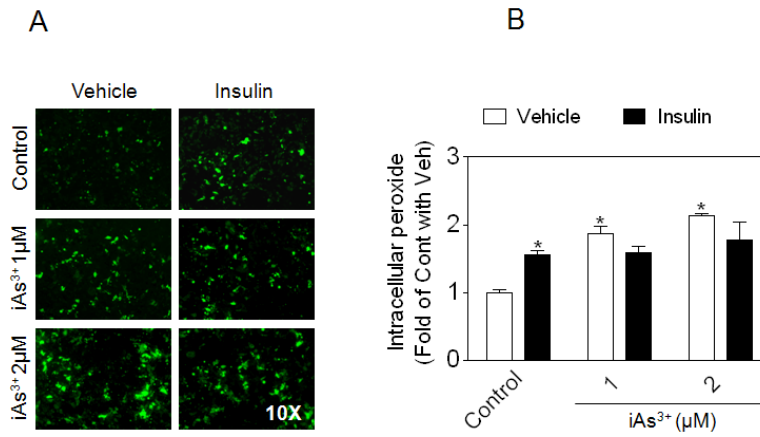
Fig. 3.
Effect of iAs^{3+} exposure on insulin-stimulated peroxide production in 3T3-L1 adipocytes. (A) Following a 7-day exposure to iAs^{3+}, intracellular peroxide level was determined using CM-H$_2$DCFDA under basal (no insulin) and insulin-challenged condition (50 ng/ml insulin; 30 min). (B) Quantification of (A). n = 2-5. * $p < 0.05$ vs. Control with vehicle.
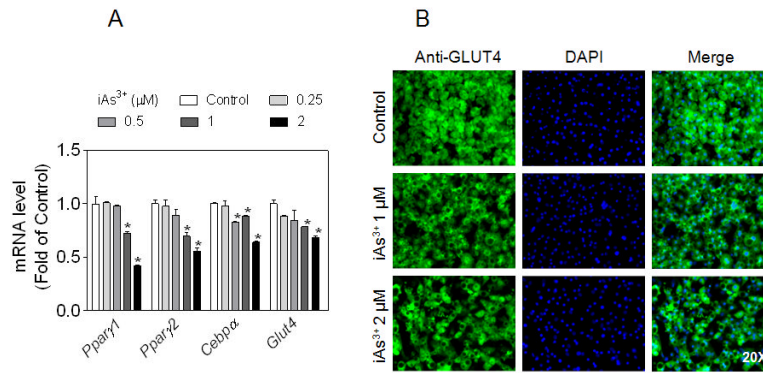
Fig. 4.
Effect of prolonged iAs^{3+} exposure on the expression of adipogenic genes and glucose transporter GLUT4 in 3T3-L1 adipocytes. Cells were exposed to low-level iAs^{3+} for 7 days. (A) Gene expression of *Pparγ*, *Cebpα* and *Glut4*. n = 3. *$p < 0.05$ vs. Control. (B) Representative images of immunostaining of GLUT4. Left panels, immunofluorescent images of GLUT4; Middle panels, nuclear DAPI staining; Right panels, merged images of GLUT4 and DAPI.