Sodium arsenite induces orphan nuclear receptor SHP gene expression via AMP-activated protein kinase to inhibit gluconeogenic enzyme gene expression

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Sodium arsenite has been demonstrated to alter the expression of genes associated with glucose homeostasis in tissues involved in the pathogenesis of type 2 diabetes; however, the underlying molecular mechanism has not been fully elucidated yet. In this study, we report that the sodium arsenite-induced gene expression of the small heterodimer partner (SHP; NR0B2), an atypical orphan nuclear receptor, regulates the expression of hepatic gluconeogenic genes. Sodium arsenite augments hepatic SHP mRNA levels in an AMP-activated protein kinase (AMPK)-dependent manner. Sodium arsenite activated AMPK and was shown to perturb cellular ATP levels. The arsenite-induced SHP mRNA level was blocked by adenoviral overexpression of dominant negative AMPK (Ad-dnAMPKα) or by the AMPK inhibitor compound C in hepatic cell lines. We demonstrated the dose-dependent induction of SHP mRNA levels by sodium arsenite and repressed the forskolin/dexamethasone-induced gene expression of the key hepatic gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Ad-dnAMPKα blocked the repressive effects of arsenite-induced SHP on PEPCK and G6Pase. Sodium arsenite induced the promoter activity of PEPCK and G6Pase, and this repression was abolished by small interfering (si)RNA SHP treatments. The knockdown of SHP expression by oligonucleotide siRNA SHP or adenoviral siRNA SHP released the sodium arsenite-mediated repression of forskolin/dexa-

methasone-stimulated PEPCK and G6Pase gene expression in a variety of hepatic cell lines. Results from our study suggest that sodium arsenite induces SHP via AMPK to inhibit the expression of hepatic gluconeogenic genes and also provide us with a novel molecular mechanism of arsenite-mediated regulation of hepatic glucose homeostasis.

small heterodimer partner; phosphoenolpyruvate carboxykinase; glucose-6-phosphatase

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associated with these metabolic pathways (6, 27, 32). AMPK activation induced by the AMPK activators 5-aminimidazole-4-carboxamide 1-β-n-ribofuranoside (AICAR) and metformin was previously demonstrated to inhibit the expression of two pivotal hepatic gluconeogenic genes, PEPCK and G6Pase, which, in turn, suppress gluconeogenesis (1, 14, 23).

Sodium arsenite is a reducing agent that stimulates a variety of signaling molecules generally associated with the cellular stress response (7, 23, 30). This chemical has been previously reported to exhibit insulin-mimetic effects on glucose homeostasis. Sodium arsenite also has been demonstrated to activate AMPK (23). Contradictory results from previous studies associated with arsenite also tend to be reflective of its effects on insulin sensitivity, resulting in modifications of the expression of a variety of genes involved in insulin resistance (31). The effects of arsenite on glucose homeostasis have been studied principally in the context of the pancreas, and only a limited amount of information is currently available regarding its effects in the liver. Reports from a variety of studies on the effects of arsenite have demonstrated the involvement of various signaling pathways in the alteration of arsenite-mediated gene expression in the regulation of glucose and lipid metabolism (31). In vivo studies conducted with chick embryos have demonstrated the repression of dexamethasone-induced PEPCK gene expression by arsenic (8, 11), further demonstrating its involvement in the regulation of one of the key gluconeogenic enzyme genes. Interestingly, reports tend to indicate possible “nontoxic” effects of high doses of arsenite, compared with what can be observed with short-term low concentrations (15), suggesting that high doses of arsenite may activate the mechanisms required for the maintenance of accurate insulin signaling and the proper maintenance of glucose homeostasis.

In this study, we have demonstrated that sodium arsenite induces SHP mRNA levels in hepatocytes and that this induction of SHP by arsenite is mediated via AMPK. SHP induction by sodium arsenite caused the repression of the hepatic gluconeogenic enzyme genes PEPCK and G6Pase, and the blockade of SHP or AMPK inhibits this sodium arsenite-mediated repression mechanism. Overall, we suggest a novel molecular mechanism exploited by sodium arsenite, which mimics the effects of insulin on hepatic gluconeogenesis and may possibly contribute to the maintenance of glucose homeostasis.

MATERIALS AND METHODS

Chemicals. Sodium arsenite (sodium metaarsenite; Wako, Osaka, Japan), dexamethasone (Sigma, St. Louis, MO), forskolin (Sigma), and insulin (Norvolin R; Green Cross, Seoul, Korea) were purchased and dissolved in the recommended solvents.

Cell culture and transient transfection assays. HepG2 (human hepatoma) and H4IIE (rat hepatoma) cell lines were cultured with Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hy- clone, Logan, UT) and antibiotics in a humidified atmosphere containing 5% CO2 at 37°C. AML12 (mouse hepatocyte) cells were cultured with ITS (insulin-transferrin-selenium; GIBCO-BRL) and dexamethasone (40 ng/ml; Sigma) and antibiotics in a humidified atmosphere containing 5% CO2 at 37°C. Transient transfection assays were conducted using LipofectAmine 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. The total amount of DNA was adjusted to 1 μg/well via the addition of the appropriate amount of pcDNA3 empty vector, and 0.2 μg of cytomegalovirus β-galactosidase plasmids were cotransfected as an internal control. After 18 h of transfection, the cells were serum starved for 24 h, followed by 6 h of pretreatment with forskolin/dexamethasone and 3 h of incubation in the presence or absence of sodium arsenite, followed by harvesting. The luciferase activity was measured and normalized to the β-galactosidase activity. All transfections were performed in accordance with the same protocols, unless otherwise stated. The data are representative of a minimum of three to five independent experiments. The potential toxicity of 3 h of sodium arsenite treatment in H4IIE cells was analyzed as previously described. The potential toxic effects on HepG2 and AML12 cells incubated with sodium arsenite for the indicated times were analyzed by determining the cell number after harvest under a light microscope in several fields of view.

Plasmids and DNA constructions. The oligonucleotide duplexed small interfering (si)RNAs corresponding to scramble (siSHP I) and SHP (siSHP II) (13) and the plasmids encoding for the dominant negative mutant AMPK (dn-AMPK) and the constitutively active form of AMPK (ca-AMPK) have been described previously (19). The reporter plasmids encoding for the human G6Pase promoter (–1,227/+57) and the rat PEPCK promoter (–2,367/+73) were generously provided by Dr. D. Schmoll (25) and Dr. R. W. Hanson (17), respectively. Mouse G6Pase cDNA was purchased from Korea UniGene Information (KUGI, Seoul, Korea), and mouse PEPCK cDNA was provided by Dr. J. B. Kim (Department of Biological Sciences, Seoul, Korea). Rat and mouse SHP cDNAs (14) were prepared as described previously. pcDNA3/HA-HNF-3β cDNA was provided by Dr. Yoon-Kwang Lee (20), and pcDNA3/HA-HNF-3β cDNA was described previously (13).

Isolation and culture of rat primary hepatocytes. Rat primary hepatocytes were isolated from the liver of 7-wk-old male Sprague-Dawley rats as described previously (14). Cultured hepatocytes were treated with sodium arsenite (1–25 μM) at the indicated time points.

Preparation of recombinant adenovirus. Adenoviruses encoding for full-length human SHP (Ad-SHP) (13), c-Myc-tagged dn-AMPKα (Ad-dnAMPKα), and α-312 AMPK, also termed ca-AMPK (in the case of mammalian expression vector) (Ad-AMPKα) (19), have all been described previously. To express the SHP gene or siRNA for SHP, cells were infected with the indicated amounts of Ad-SHP or Ad-siRNA SHP in DMEM. After 4 h of viral infection, the culture medium was exchanged with serum-starved fresh medium for further experiments.

Northern blot analysis. HepG2, H4IIE, and AML12 cell lines were maintained in the indicated media. At ~80% confluence, the cells were subjected to serum starvation (medium with 0.5% FBS). After 24 h, the cells were treated with sodium arsenite (indicated doses), forskolin (10 μM), and dexamethasone (100 nM) and then infected with adenoviral vectors encoding for SHP, siRNA SHP, AMPK, or dn-AMPKα at the indicated times. The cells were subsequently harvested at the indicated times, and the total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Aliquots of 20 μg of total RNA from each sample were used for Northern blot analysis as previously described (29). The probe labeling of each of the cDNAs for SHP, PEPCK, G6Pase, and GAPDH with [α-32P]dCTP was performed using a random-primer DNA-labeling system (Amersham Biosciences, Little Chalfont, UK). The expression of all transcripts was normalized to GAPDH levels. All Northern blot analyses were performed identically unless otherwise stated. The data are representative of a minimum of three to five independent experiments.

RT-PCR analysis. RT-PCR analyses using RNA samples from rat primary hepatocytes were performed as described previously (13). The bands were analyzed densitometrically. The data are representative of a minimum of three to five independent experiments.

Western blot analysis. H4IIE cells were treated with sodium arsenite (for the indicated time periods) and AICAR for 12 h, after which
the cells were then harvested and homogenized in IPH lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 100 mM PMSF, 1 M DTT, 1 mg/ml leupeptin, and 1 mg/ml aprotinin). The cell lysates (50 μg/lane) were separated via SDS-PAGE on an 8% gel, and the proteins were transferred to Hybond-C extra nylon membranes. The membranes were then probed with a polyclonal phospho-acetyl-CoA carboxylase (p-ACC) antibody (Ser79; no. 3661; Cell Signaling Technology, Beverly, MA), polyclonal ACC antibody (no. 3662; Cell Signaling Technology), monoclonal phospho-AMPKα antibody (Thr172; no. 2535; Cell Signaling Technology), and a polyclonal AMPKα antibody (no. 2532; Cell Signaling Technology). Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies and an ECL Western blotting detection kit (Amersham Bioscience, Rothkreuz, Switzerland) were used for protein detection.

AMPK assay. The AMPK assay was performed using the SAMS peptide phosphorylation assay kit from Upstate Biotechnology in accordance with the manufacturer’s protocol. In brief, H4IIE cells were cultured in serum-free medium for 18 h before chemical treatments. Chemicals were added directly to the cell culture dishes, and cells were incubated for 5 min for sodium arsenite or 2 h for AICAR at 37°C. Culture medium was quickly removed, and cells were washed once with ice-cold PBS and harvested in 50 mM Tris·HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM benzamidine, 4 μg/ml soybean trypsin inhibitor, 1 mM PMSF, 250 mM mannotol, and protease inhibitor tablets (Roche). Cellular debris was removed by centrifugation at 10,000 g at 4°C for 20 min, and the supernatant was snap-frozen in liquid nitrogen. Samples were stored at −70°C before AMPK activity assays. Proteins in the supernatant were concentrated by polyethylene glycol (PEG) 8000 precipitation, and the AMPK reaction was performed for 10 min at 30°C with 20 μM SAMS peptide, 10 μCi of [γ-32P]ATP, and a 10-μg protein sample. The reaction mixture was then spotted on P81 phosphocellulose paper (Upstate Biotechnology), which was washed with 0.75% phosphoric acid and acetone, and the radioactivity of phosphorylated SAMS peptide was quantified by scintillation counting.

Measurement of ATP concentration. H4IIE cells were seeded in a 96-well plate. Cells were exposed to sodium arsenite doses for the times indicated. ATP concentration was estimated via luciferase activity using the ATP bioluminescence assay kit (Roche Applied Bioscience, Rothkreuz, Switzerland). Results are expressed as the degree of change of control cells (set as unity) not exposed to any chemicals.

Statistical analysis. Data are means ± SE. Analysis of variance (ANOVA) was used to determine significant differences, followed by Duncan’s multiple comparison tests. All experiments were performed at least three times. Data calculation and statistical analysis were performed using GraphPad Prism 4.0 software. Two-way ANOVA analysis for repeated measures and Student’s t-test for unpaired data were used as appropriate to detect any significant differences. Significance was accepted at the P < 0.05 level.

RESULTS

Sodium arsenite increases mRNA levels of SHP in hepatic cells. Sodium arsenite has been previously reported to repress the expression of PEPCK gene (23, 30). However, the molecular mechanisms underlying this process have not been fully elucidated yet. To determine whether sodium arsenite regulates SHP mRNA levels, we performed Northern blot analysis on sodium arsenite-treated hepatoma cell lines (HepG2 and H4IIE), nontransformed mouse hepatocytes (AML12), and rat primary hepatocytes. The levels of SHP mRNA were significantly induced by sodium arsenite in both time- and dose-dependent manners in all of the tested cell lines and primary hepatocytes (Fig. 1, A–D). Sodium arsenite treatment induced a rapid induction of SHP mRNA expression by three- to sixfold in all tested cell lines. SHP expression declined with longer treatment periods, particularly in the H4IIE cells (Fig. 1B), whereas in the cases of the HepG2 and AML12 cells as well as primary hepatocytes, this effect appeared to be more sustained (Fig. 1, A, C, and D). Dose-dependent sodium arsenite treatments (1–25 μM) showed a similar pattern of increase in SHP mRNA levels in all the cell lines and reached a peak at a sodium arsenite concentration of 25 μM within 1–3 h. The changes in SHP mRNA levels to the internal loading control GAPDH were quantified for time-dependent (Fig. 1E) and dose-dependent (Fig. 1F) arsenite treatments. Sodium arsenite exerted no effects on cell viability at the times and concentrations examined in any of these cell lines. Furthermore, arsenite had no effect on endogenous GAPDH expression. These results indicate that sodium arsenite induces SHP gene expression in hepatic cell lines via an increase in SHP mRNA levels.

Sodium arsenite activates AMPK. Sodium arsenite is a reducing agent that is commonly associated with cellular stresses resulting in alterations in the cellular ATP/AMP ratio, thereby activating the AMPK signaling pathway (23). To evaluate the involvement of AMPK activation in sodium arsenite-mediated SHP gene regulation, we initially assessed the effects of sodium arsenite on AMPK activation via Western blot analysis, to check the levels of AMPK phosphorylation in the presence or absence of sodium arsenite and AICAR, an AMPK activator, using anti-phospho-Ser79 ACC antibody and anti-phospho-Thr172 AMPK antibody. Sodium arsenite increased ACC and AMPKα phosphorylation levels (Fig. 2, A–D) in all the tested hepatic cell lines. Sodium arsenite induced a rapid phosphorylation, within 5–15 min, and the phosphorylation levels of both ACC and AMPKα declined gradually after that. The change in the degree of activity of the phosphorylation levels of ACC and AMPKα on arsenite treatment were normalized to the levels of the total ACC (t-ACC) and total AMPKα (t-AMPKα) protein levels, respectively (Fig. 2, E and F). These results show consistent and rapid phosphorylation and activation of AMPKα as well as its downstream target, ACC, as a result of sodium arsenite treatment.

To further confirm the sodium arsenite-mediated activation of AMPK, we examined the effects of Ad-dnAMPKα overexpression on ACC phosphorylation (p-ACC) levels by arsenite treatment in H4IIE cells. Cells overexpressing Ad-dnAMPKα were treated with sodium arsenite or AICAR, and p-ACC levels were shown to be drastically reduced in the presence of Ad-dnAMPKα compared with arsenite or AICAR treatments alone (Fig. 3A). This result demonstrates the efficacy of the dominant negative form of AMPKα and also confirms the activation of AMPK by sodium arsenite. We then attempted to elucidate the mechanism underlying the sodium arsenite-induced AMPK activation in H4IIE cells. AMPK activity was assessed in cells exposed to various doses of arsenite (1–100 μM) for 5 min. AICAR was used as a positive control for 2 h. As expected, a robust increase of AMPK activity was observed with AICAR, and, most importantly, arsenite strongly activated AMPK (Fig. 3B) in a dose-dependent manner, with the highest activity being reached at 25 μM. Sodium arsenite induces AMPK activity at a comparable level to AICAR. Finally, we investigated whether arsenite-induced AMPK activation is associated with a decrease in ATP concentration. A significant decrease (by 40%) of ATP levels was observed in
cells following 5 min of arsenite treatment (Fig. 3C) and at concentrations of 25 μM and higher (Fig. 3D). AICAR exerted no significant effects on cellular ATP levels. Collectively, our results indicate that AMPK activation by sodium arsenite may be mediated by a decrease in intracellular ATP concentrations.

Sodium arsenite increases mRNA levels of SHP and represses PEPCK and G6Pase gene expression. To determine the involvement of sodium arsenite-mediated SHP gene expression in the regulation of hepatic gluconeogenesis, we initially examined the effects of sodium arsenite on the PEPCK and G6Pase gene promoter in AML12 cells. Sodium arsenite (1–25 μM) repressed the forskolin/dexamethasone-stimulated promoter activity of PEPCK and G6Pase in a dose-dependent manner, with a significant effect observed at 25 μM, which was reminiscent of the decrease observed in insulin-treated cells (Fig. 4A). To confirm this repression of PEPCK and G6Pase at the mRNA level by sodium arsenite via the induction of SHP, we performed Northern blot analysis to determine the expression of PEPCK, G6Pase, and SHP in response to sodium arsenite treatment in the H4IIE cells. Pretreatment of cells with forskolin/dexamethasone induced PEPCK and G6Pase mRNA levels. Dose-dependent treatments with sodium arsenite (1–25 μM) induced a dramatic increase in SHP mRNA levels and profoundly repressed the forskolin/dexamethasone-induced mRNA levels of PEPCK and G6Pase (Fig. 4B) in a manner reminiscent of that observed with insulin-treated cells. Results were statistically significant and reproducible at higher doses (10 and 25 μM) compared with 1 μM treatment (Fig. 4C). Together, these results show that sodium arsenite-induced SHP gene expression is associated with the repression of the SHP target genes associated with hepatic gluconeogenesis.

Activation of AMPK by sodium arsenite induces SHP mRNA levels to inhibit PEPCK and G6Pase gene expression. To evaluate the role of AMPK in sodium arsenite-induced SHP gene expression in the regulation of hepatic gluconeogenic gene expression, we initially examined the effects of the pharmacological inhibitor of AMPK, compound C, as well as Ad-dnAMPKα on the sodium arsenite-mediated induction of SHP mRNA levels in H4IIE cells (Fig. 5, A and B). Pretreatment of cells with compound C profoundly repressed the increase of SHP mRNA levels induced by sodium arsenite (Fig. 5A). Similarly, the overexpression of Ad-dnAMPKα significantly repressed the sodium arsenite-mediated increase of SHP mRNA levels in the H4IIE cells (Fig. 5B). Ad-siRNA SHP overexpression also significantly blocked the arsenite-mediated induction of SHP mRNA levels, and this served as a control experiment to confirm the specificity of SHP induction by arsenite (Fig. 5B). Collectively, these results indicate that sodium arsenite increases SHP mRNA levels via AMPK activation.

Next, to determine the mediation of AMPK in the induction of SHP mRNA levels by arsenite and subsequent regulation of hepatic gluconeogenic genes, we performed Northern blot analysis with H4IIE cells. Adenoviral vectors expressing the constitutively active form of AMPK (Ad-

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**Fig. 1.** Sodium arsenite (NaArs) increases mRNA levels of the small heterodimer partner (SHP). A–D: HepG2 (A), H4IIE (B), AML12 (C), and rat primary hepatocytes (RPH; D) were cultured for 24 h under serum-starved conditions. The cells were then treated with various doses (1–25 μM) of NaArs for 6 h or for various time periods (1–6 h). SHP gene expression was verified via Northern blot analysis and normalized to GAPDH expression. Data are representative of 3 independently performed experiments. E and F: quantitative representation of the time (E) and dose-dependent (F) changes in SHP mRNA levels was normalized to GAPDH expression and expressed as the degree of change compared with GAPDH. Data are means ± SE. *P < 0.05; #P < 0.001 compared with expression at 0 h.
AMPKα increased the SHP gene expression in a manner similar to that of the cells treated with Ad-SHP (Fig. 5, C and D) or arsenite treatments alone and repressed the forskolin/dexamethasone-induced mRNA levels of PEPCK and G6Pase in a pattern identical to that of dose-dependent sodium arsenite treatment in the H4IIE cells. To further confirm that the sodium arsenite-dependent increase of SHP mRNA levels and the subsequent repression of PEPCK and G6Pase was mediated by AMPK, we performed Northern blot analysis with H4IIE cells. Cells were stimulated with forskolin/dexamethasone treatments and pretreated for 1 h with compound C before sodium arsenite treatment. Repression of PEPCK and G6Pase along with the induction of SHP mRNA levels by arsenite was significantly reversed on pretreatments with compound C (Fig. 5, E and F). Overall, these results suggest that AMPK plays a crucial role in sodium arsenite-mediated induction of SHP and, consequently, the downregulation of the gluconeogenic target genes of SHP.

Inhibition of AMPK activity reverses sodium arsenite-induced SHP gene expression and inhibition of PEPCK and G6Pase gene expression. To confirm the importance of AMPK in sodium arsenite-induced SHP gene expression in the regulation of hepatic gluconeogenic gene activity and expression, we examined the effects of expression vectors encoding for ca-AMPK and dn-AMPK or compound C on AML12 cells transiently transfected with PEPCK and G6Pase wild-type promoter constructs and stimulated via forskolin/dexamethasone pretreatment. Sodium arsenite repressed the promoter activity of forskolin/dexamethasone-stimulated PEPCK and G6Pase in a pattern similar to the repression observed in conjunction with ca-AMPK. The promoter activity of both PEPCK and G6Pase was recovered significantly upon cotransfections with dn-AMPK or pretreatment with compound C (Fig. 6, A and B). This indicates that AMPK activation mediates the sodium arsenite-mediated repression of the PEPCK and G6Pase promoters.

Next, to further confirm the above results at the mRNA level, we infected H4IIE cells with Ad-dnAMPKα for 24 h at different multiplicities of infection (MOI). The overexpression of Ad-dnAMPKα significantly repressed the increase in SHP mRNA levels by sodium arsenite. Consequently, the repression of forskolin/dexamethasone-induced PEPCK and G6Pase mRNA levels by sodium arsenite-induced SHP mRNA levels was dramatically reversed (Fig. 6, C and D). Collectively, these results demonstrate that AMPK mediates sodium arsenite-induced SHP gene expression, thus inhibiting the expression of hepatic gluconeogenic genes.

Sodium arsenite represses the activity of PEPCK and G6Pase gene promoter via SHP. As reported previously, the promoter activity of PEPCK and G6Pase is regulated by the transcription factors HNF-4α (36) and HNF-3β (14), respectively. SHP also has been reported to inhibit the HNF-4α-mediated transcriptional upregulation (36) of PEPCK as well as the HNF-3β-mediated transactivity of the G6Pase promoter (14). To determine the role of sodium arsenite-induced SHP expression in the HNF-4α and HNF-3β-mediated transcriptional regulation of the PEPCK and G6Pase promoters, we examined the effects of sodium arsenite on the transactivation of HNF-4α and HNF-3β in AML-12 cells. Sodium arsenite repressed the HNF-4α transactivation of PEPCK promoter activity in a pattern similar to that observed with SHP, and the knockdown of endogenous SHP gene expression significantly reversed the sodium arsenite-mediated repression of PEPCK promoter activity (Fig. 7A). Similarly, in the presence of HNF-3β, sodium arsenite and SHP significantly repressed G6Pase promoter activity, and oligonucleotide siRNA SHP
Fig. 3. NaArs activates AMPK and lowers cellular ATP levels. A: H4IIE cells were infected with or without the adenovirus dominant negative form of AMPK (Ad-dnAMPKα) tagged with green fluorescent protein (GFP) at a multiplicity of infection (MOI) of 50 for 24 h. After 24 h, the cells were serum starved for an additional 24 h, followed by 5 min of treatment with NaArs (25 μM) or 24 h of treatment with AICAR (500 μM) in the presence or absence of Ad-dnAMPKα. The cell lysates were prepared, and the total proteins were resolved on 8% SDS-PAGE gel. Phosphorylation of the downstream target of AMPK and total ACC levels were determined using anti-phospho-Ser79 ACC (p-ACC) and total ACC (t-ACC) antibody, respectively. Data are means ± SE representative of at least 3 independently performed experiments. Western blot results were quantified as the degree of change of p-ACC over t-ACC levels. *P < 0.05 compared with untreated samples. **P < 0.05 compared with NaArs/AICAR-treated samples.

B: AMPK activity in H4IIE cells was assayed in crude extracts against the synthetic peptide SAMS, as described in MATERIALS AND METHODS. H4IIE cells were serum starved for 24 h, followed by incubation with NaArs (25 μM) for 5 min or AICAR (500 μM) for 2 h before cell lysis and protein extraction. Data are means ± SE representative of at least 3 independent determinations. *P < 0.05 compared with untreated samples. C and D: H4IIE cells were serum starved for 24 h, followed by treatments with NaArs for varying time points from 1 to 10 min (C) and varying doses from 1 to 25 μM (D). Cells were treated with AICAR (500 μM) for 12 h. Cells were then lysed according to the manufacturer’s instructions. Total intracellular ATP was quantified by luciferase. Results are expressed as the decrease in levels of intracellular ATP, with untreated cells set as unity, normalized to the total protein level and correspond to at least 3 independent experiments. *P < 0.05; #P < 0.001 compared with untreated cells.

In an effort to confirm these results (Fig. 7) at the mRNA level, we performed Northern blot analysis in rat primary hepatocytes. Cultured primary hepatocytes were infected with Ad-dnAMPKα or Ad-siRNA SHP for 24 h (MOI = 50), followed by forskolin/dexamethasone and sodium arsenite treatments (Fig. 8, C and D). The PEPCK and G6Pase mRNA levels were increased in response to forskolin/dexamethasone treatment. Arsenite treatment induced SHP gene expression and significantly repressed PEPCK and G6Pase gene expression. This arsenite-mediated induction of SHP gene expression and subsequent repression of PEPCK and G6Pase was drastically reversed on Ad-dnAMPKα or Ad-siRNA SHP-treated hepatocytes (Fig. 8D). Collectively, these results show that the AMPK-mediated induction of SHP gene expression by sodium arsenite is con-

SODIUM ARSENITE INDUCES SHP GENE EXPRESSION

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sistent in all hepatic cells and performs a significant role in the downregulation of hepatic gluconeogenesis.

**DISCUSSION**

In this study, we have shown that sodium arsenite functions as an inducer of SHP mRNA levels via AMPK activation and inhibits the expression of the two key hepatic gluconeogenic genes PEPCK and G6Pase. The sodium arsenite-mediated repression of hepatic gluconeogenesis by SHP was blocked by the knockdown of endogenous SHP expression in hepatic cell lines with the use of an oligonucleotide duplex and adenoviral expression vectors designed against SHP. Moreover, the inhibitory effects of sodium arsenite-induced SHP gene on the expression of the two key gluconeogenic enzyme genes PEPCK and G6Pase was released by dominant negative AMPK as well as by the AMPK inhibitor compound C. Overall, our result indicates that sodium arsenite inducers of SHP gene expression. Previous reports have proposed that sodium arsenite elicits cellular stress responses in certain cell types, thereby resulting in an increase in the AMP/ATP ratio and, consequently, AMPK (23). Arsenite treatments in H4IIE cells were also determined to activate AMPK to a level equivalent to that achieved with AICAR treatment (23). Sodium arsenite also has been demonstrated in previous studies to affect gluconeogenic gene expression, including H2O2, also have been demonstrated to affect gluconeogenic gene expression reminiscent of the effects.
Fig. 5. NaArs activates AMPK and increases SHP mRNA levels to inhibit PEPCK and G6Pase gene expression. A: H4IIE cells were maintained in serum-starved conditions for 24 h, followed by pretreatment with the AMPK inhibitor compound C for 1 h. After that, cells were further treated with or without NaArs (25 μM) for 1 h in the continuing presence of compound C. SHP mRNA levels were confirmed via Northern blot analysis and normalized to GAPDH expression. Data are means ± SE representative of 3 independently performed experiments. *P < 0.05 compared with basal expression. **P < 0.05 compared with NaArs-induced expression.

B: H4IIE cells were infected with adenoviral vector expressing siRNA SHP (Ad-siSHP) tagged with GFP and with Ad-dnAMPKα tagged with GFP at a MOI of 50 for 24 h. After 24 h, the cells were further serum starved for 24 h, followed by 1 h of treatment with NaArs. SHP mRNA levels were verified via Northern blot analysis and normalized to GAPDH as an internal loading control. Data are means ± SE representative of at least 5 independently performed Northern blot analyses. *P < 0.05 compared with basal expression. #P < 0.001 compared with NaArs-induced expression.

C: H4IIE cells were infected with Ad-SHP or Ad-AMPKα at a MOI of 50 for 24 h. After 24 h, the cells were further serum starved for 24 h, followed by treatments with Fsk/Dexa for an additional 6 h with or without insulin (10 nM) treatment for 1 h. PEPCK, G6Pase, and SHP mRNA levels were verified via Northern blot analysis and normalized to GAPDH as an internal loading control. Data are representative of at least 3 independently performed Northern blot analyses. *P < 0.05 compared with Fsk/Dexa-stimulated expression. **P < 0.05; bP < 0.05 compared with NaArs-treated expression.

D: quantitative representation of the changes in PEPCK, G6Pase, and SHP mRNA levels normalized to GAPDH mRNA levels expressed as the degree of change compared with GAPDH. Data are means ± SE. *P < 0.05; #P < 0.001 compared with basal expression. aP < 0.05 compared with Fsk/Dexa-stimulated expression. **P < 0.05; bP < 0.05 compared with NaArs-treated expression.
The proposed upstream signal transduction pathway involved was shown to be both phosphoinositide 3-kinase (PI 3-kinase)- and mitogen-activated protein kinase (MAPK)-independent in nature (30). These results indicated the involvement of the activation of other signaling molecules involved in the cellular stress conditions associated with either arsenite or \( \text{H}_2\text{O}_2 \). Thus the signaling pathway exploited by arsenite or other agents that elicit a cellular stress response are distinct from those exploited by insulin for the regulation of PEPCK and G6Pase gene expression in hepatocytes. Among other insulin-mimetic agents, compounds of trace elements such as vanadate and selenate have been touted to exhibit antidiabetic effects both in vitro and in vivo (24, 28, 29). The ability of these compounds to stimulate glucose uptake and glycogen and lipid synthesis in muscle, adipose, and hepatic tissues and to inhibit gluconeogenesis via the regulation of PEPCK and G6Pase gene expression in hepatocytes. Proper in vivo studies, however, are required using knockout mice models for SHP to clarify the potential involvement in hepatic gluconeogenesis, as well as to verify the function performed by SHP activators such as arsenite in glucose metabolism. This may provide us with new insights into the functions of major transcription factors such as SHP in the regulation of gluconeogenic genes, as well as novel therapeutic approaches required for the more efficient treatment of diabetes.

AMPK is the central component of a protein kinase cascade, monitoring cellular energy charge and being activated by a rise in the cellular AMP/ATP ratio (3, 7, 9, 12). AMPK also regulates metabolism via both the direct phosphorylation of metabolic enzymes and its effects on gene expression (10, 12). In this study, we further attempted to elucidate the possible mechanism by which arsenite activates AMPK. Pathological stresses are implicated to interfere with ATP production, and in our study we have demonstrated that, indeed, arsenite causes a reduction in cellular ATP levels, suggesting a possible mechanism by which it increases the AMP/ATP ratio, subsequently activating AMPK (9).

Recently, our group (14) showed, under both in vitro and in vivo conditions, that metformin, an antidiabetic drug, activates AMPK and downregulates PEPCK and G6Pase gene expression via the upregulation of SHP gene expression. The effects of metformin on SHP gene regulation and the physi-
logical outcomes observed further support our results concerning arsenite-mediated induction of SHP gene expression via AMPK and the repression of PEPCK and G6Pase gene expression. Both studies concern the effects of arsenite as well as metformin on gluconeogenesis, thereby suggesting that induction of SHP gene expression via activation of AMPK as a result of these treatments, though by way of contrast to metformin-mediated SHP induction, arsenite appears to upregulate SHP gene expression at a considerably earlier time point. As previously mentioned, the possibility of protein stabilization or the phosphorylation of SHP by arsenite via AMPK cannot currently be ruled out as a possible mechanism of this AMPK-mediated activation of SHP by arsenite. This consistency in the results obtained with arsenite compared with our previous report dealing with metformin tempts us to predict that SHP may be one of the primary targets of AMPK, providing us with a more detailed notion of the relevant molecular mechanisms, thereby resulting in the identification of other potential SHP regulators.

As mentioned earlier, SHP predominantly functions as a transcriptional corepressor for a diverse group of transcription factors, including members of the nuclear receptor superfamily and nonnuclear receptor transcription factors (4, 21). Previous reports have resulted in the identification of a large number of potential SHP targets known to regulate the PEPCK and G6Pase promoters. The HNF family of transcription factors has long been considered to be a major transcriptional activator of PEPCK and G6Pase (13, 34). Other important targets of SHP in glucose metabolism are the forkhead transcription factor FOXO1, the basic loop-helix-loop protein BETA2, and the aryl receptor nuclear translocator ARNT.

The involvement of other signaling pathways resulting in SHP induction and a consequent regulation of hepatic gluconeogenesis cannot be completely ruled out. Another important aspect of this AMPK-mediated SHP regulation is the possibility of the involvement of other transcription factors that may regulate the SHP promoter via AMPK activation. This search for the putative transcription factors that may possibly mediate this activity of AMPK on SHP should provide us with a more detailed notion of the relevant molecular mechanisms, thereby resulting in the identification of other potential SHP regulators.

Other reported activators of AMPK, including natural plant products such as berberine (22) or epigallocatechin gallate (EGCG) (5), have been reported to improve glucose tolerance and the reduction of body weight, as well as the inhibition of hepatic gluconeogenesis. These AMPK activators may comprise yet another group of therapeutic agents for the treatment of type 2 diabetes, acting via SHP. Although PI 3-kinase has also been associated with the insulin-mimetic effects of EGCG (22), and our results indicate that the arsenite-mediated induction of SHP occurs in an AMPK-dependent manner, the involvement of other signaling pathways resulting in SHP induction and a consequent regulation of hepatic gluconeogenesis cannot be completely ruled out. Another important aspect of this AMPK-mediated SHP regulation is the possibility of the involvement of other transcription factors that may regulate the SHP promoter via AMPK activation. This search for the putative transcription factors that may possibly mediate this activity of AMPK on SHP should provide us with a more detailed notion of the relevant molecular mechanisms, thereby resulting in the identification of other potential SHP regulators.

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gluconeogenic gene promoter regulation such as peroxisome proliferator-activated protein receptor-γ coactivator-1α (PGC-1α) and p300/cAMP-response element binding protein (p300/CBP) (4, 21). SHP also has been reported to downregulate the forkhead transcription factor-induced promoter activity of PGC-1α (4, 21). In this study, we assessed whether the HNF-mediated transcriptional activation of PEPCK and G6Pase could be repressed by sodium arsenite-induced SHP. Our results showed that sodium arsenite inhibited both the HNF-4α- and HNF-3β-mediated transcriptional activity of PEPCK and G6Pase. The inhibition of endogenous SHP via the siRNA approach dramatically recovered the promoter activity of both PEPCK and G6Pase from arsenite-mediated repression. A previous report also demonstrated HNF-4α to be a direct target of AMPK (18). AMPK was shown to modulate HNF-4α protein levels in both pancreatic β-cells and hepatocytes and thereby down-regulates HNF-4α target genes. Overall, these results show that the arsenite-mediated induction of SHP inhibits HNF-mediated transcriptional activity of gluconeogenic gene promoter activity, although the possibility of direct targeting of HNF-4α by arsenite, in an AMPK-dependent manner, thus resulting in a decrease in the HNF-4α protein levels and subsequent activity, cannot be completely ruled out.

Glucagon (via the second messenger, cAMP) and glucocorticoids (via glucocorticoid receptor) are the two major hormones known to regulate the expression of hepatic gluconeogenic genes (2, 16). Our data show that sodium arsenite inhibited the forskolin/dexamethasone-induced gene expression of PEPCK and G6Pase. This inhibition was accompanied by a marked increase in SHP mRNA levels, thus demonstrating the involvement of SHP in arsenite-mediated repression. A previous report also demonstrated HNF-4α to be a direct target of AMPK (18). AMPK was shown to modulate HNF-4α protein levels in both pancreatic β-cells and hepatocytes and thereby down-regulates HNF-4α target genes. Overall, these results show that the arsenite-mediated induction of SHP inhibits HNF-mediated transcriptional activity of gluconeogenic gene promoter activity, although the possibility of direct targeting of HNF-4α by arsenite, in an AMPK-dependent manner, thus resulting in a decrease in the HNF-4α protein levels and subsequent activity, cannot be completely ruled out.

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other corepressors due to the arsenite-mediated activation of SHP may help to explain the repression of gluconeogenic enzyme gene promoter activity by arsenite, a phenomenon that clearly warrants more detailed study.

In summary, we suggest that the insulin-mimetic effects of sodium arsenite on hepatic gluconeogenic gene expression via AMPK phosphorylation and activation are mediated by the induction of the downstream target SHP, which represses PEPCK and G6Pase promoter activity and mRNA levels. Because these effects of sodium arsenite are dependent on the activation of AMPK followed by the upregulation of its downstream target SHP, our findings strengthen the view that the identification of other agents resulting in the pharmacological activation of AMPK may constitute an effective method for the challenge of type 2 diabetes.

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GRANTS

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