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Functional RNA Interference (RNAi) Screen Identifies System A Neutral Amino Acid Transporter 2 (SNAT2) as a Mediator of Arsenic-induced Endoplasmic Reticulum Stress

**Background:** Arsenic causes ER stress, but the underlying mechanisms remain incompletely understood.

**Results:** A genome-wide RNAi screen identifies human genes that mediate arsenite-induced ER stress.

**Conclusion:** Up-regulation of the amino acid transporter SNAT2 links mTOR activation and ER stress during arsenite exposure.

**Significance:** Our study helps to better understand arsenic-related human diseases, such as cancer and diabetes.

Exposure to the toxic metalloid arsenic is associated with diabetes and cancer and causes proteotoxicity and endoplasmic reticulum (ER) stress at the cellular level. Adaptive responses to ER stress are implicated in cancer and diabetes; thus, understanding mechanisms of arsenic-induced ER stress may offer insights into pathogenesis. Here, we identify genes required for arsenite-induced ER stress response in a genome-wide RNAi screen. Using an shRNA library targeting ~20,000 human genes, together with an ER stress cell model, we performed flow cytometry-based cell sorting to isolate cells with defective response to arsenite. Our screen discovered several genes modulating arsenite-induced ER stress, including sodium-dependent neutral amino acid transporter, SNAT2. SNAT2 expression and activity are up-regulated by arsenite, in a manner dependent on activating transcription factor 4 (ATF4), an important mediator of the integrated stress response. Inhibition of SNAT2 expression or activity or deprivation of its primary substrate, glutamine, specifically suppressed ER stress induced by arsenite but not tunicamycin. Induction of SNAT2 is coincident with activation of the nutrient-sensing mammalian target of rapamycin (mTOR) pathway, which is at least partially required for arsenite-induced ER stress. Importantly, inhibition of the SNAT2 or the System L transporter, LAT1, suppressed mTOR activation by arsenite, supporting a role for these transporters in modulating amino acid signaling. These findings reveal SNAT2 as an important and specific mediator of arsenic-induced ER stress, and suggest a role for aberrant mTOR activation in arsenic-related human diseases. Furthermore, this study demonstrates the utility of RNAi screens in elucidating cellular mechanisms of environmental toxins.

Arsenic contamination in the environment poses a major threat to global public health. Epidemiological studies observe a link between arsenic exposure, primarily via contaminated drinking water, and the development of numerous chronic diseases, such as cancer, type 2 diabetes, and cardiovascular disease (reviewed in Refs. 1–3). The precise molecular mechanisms leading to arsenic toxicity and disease development are not well understood. Arsenite (As(III)), a toxic inorganic species found in ground water, can react with thiols, resulting in protein damage and oxidative stress (4). Consequently, arsenite elicits stress responses that counteract protein misfolding, such as induction of heat shock protein chaperones (5), ubiquitin-proteasome system components (6), and the unfolded protein response (UPR)2 (7–11).

Various stresses (e.g. glucose deprivation and oxidative stress) can disrupt endoplasmic reticulum (ER) function, leading to the accumulation of misfolded proteins in the organelle, inducing a condition called ER stress (12). To maintain organelle homeostasis, an adaptive transcriptional response program, the UPR, expands ER functional capacities, such as chaperoning and degradation, and promotes recovery from stress via the integrated stress response (reviewed in Ref. 13). Mammalian UPR is initiated by ER-localized membrane proteins, inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like ER kinase (PERK); these proteins regulate three canonical branches of the UPR. During ER stress,

\[ \text{ER stress} \rightarrow \text{UPR} \rightarrow \text{mTOR and SNAT2} \]

\[ \text{mTOR activation} \rightarrow \text{SNAT2 expression and activity} \]

\[ \text{SNAT2 upregulation} \rightarrow \text{arsenic-induced ER stress} \]

These findings reveal SNAT2 as an important and specific mediator of arsenic-induced ER stress, and suggest a role for aberrant mTOR activation in arsenic-related human diseases. More specifically, this study demonstrates the utility of RNAi screens in elucidating cellular mechanisms of environmental toxins.
the endoribonuclease function of IRE1 splices X-box-binding protein 1 (XBP1) mRNA, resulting in translation of the UPR-promoting transcription factor (14). ATF6 translocates to the nucleus during ER stress to induce expression of ER chaperones, such as GRP78 (15). PERK is an eIF-2α kinase that promotes translation of activating transcription factor 4 (ATF4) and the integrated stress response (16, 17). UPR can also promote cell death through activation of multiple proapoptotic effectors, such as proinflammatory JNK and C/EBP-homologous protein (CHOP) (reviewed in Refs. 18 and 19).

There is growing evidence that arsenic can cause ER stress. Arsenite has been shown to activate various UPR proteins, including IRE1, GRP78, ATF4, and CHOP (7–11). The eIF-2α kinase engaged during arsenite stress is HRI or PKR (20, 21) and not PERK, indicating a non-canonical mechanism of ER stress induction. Because chronic ER stress is recognized as a factor in numerous diseases, most notably diabetes (19, 22, 23) and cancer (24, 25), it is likely that ER stress and UPR activation are important contributing factors in arsenic-associated diseases. To identify the molecular machinery involved in sensing arsenic stress and engaging ER stress response pathways, we performed a genome-wide RNAi screen for genes required for arsenite-induced ER stress. One of the validated RNAi hits is sodium-dependent neutral amino acid transporter 2 (SNAT2), which is activated by arsenite stress in an ATF4-dependent manner. This arsenite-induced increase in SNAT2 activity appears to link mTOR activity and ER stress during exposure to this toxin.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Chemicals—**Human embryonic kidney HEK293 and HEK293T cells were cultured as previously described (26). Mouse 3T3L1 adipocytes were cultured and differentiated as described previously (27). Experiments on adipocytes were performed on days 8–10 after starting differentiation. All chemicals were obtained from Sigma-Aldrich.

**CHOP Promoter Reporter Construct and Cell Line Generation, Lentiviral shRNA Library, and Screening—**A human CHOP promoter construct was a gift from P. Fafournoux (28). The promoter region (−649 to +91) was extended at the 3’-end to include the complete upstream ORF that inhibits basal CHOP expression (29). The 3’-end was extended using two overlapping primers (5’- GTATGAAAGATACACTTCTCTTTTGAGAACCTCCTCCTTTAGAGGGTGTTCAGGGGCTG-3’ and 5’-CGGCATTCTGTTGGTGTAGTGATGAAAGGTACACTTCTTTG3’) in 35 sequential PCRs by standard procedures (30). This “sublibrary” of shRNAs was then transformed into supercompetent Escherichia coli (Stratagene), which produced ∼5 × 10⁶ individual bacterial colonies. shRNA plasmids were recovered from 268 clones and sequenced using vector sequencing primers. Target gene identity was determined with the genome search engine, BLAT (University of California, Santa Cruz) (31).

**Flow Cytometry Assay—**To evaluate the reporter phenotype produced by individual shRNAs, CHOP reporter cells were transduced with individual lentiviral shRNA particles. Lentiviral particles were prepared and shRNA-transduced into the reporter cell line as described above. Following puromycin selection, cells were treated with arsenite (15 μM, 16 h) and then analyzed by flow cytometry (BD LSRII). All flow cytometry data were collected using BD Diva software and analyzed with DeNovo FCS Express software.

**siRNA Transfections—**HEK293T cells were transected with 100 nM siRNA (Sigma) (guide strands, 5’-AAUUGGCACAGCAUAGACAG (si1) or 5’-ACUAUGAGAGGUAGCUG (si2)) targeting SNAT2 mRNA (accession NM_018976). Cells were also transected with siRNA (Sigma) (guide strand, 5’-AAUCUGUCCGGAGAGG (targeting ATF4 mRNA (accession NM_001675)) or non-targeting siRNA control (Sigma, Mission Universal Negative Control 1), using Dharmafect 1 transfection reagent (Dharmacon), according to the manufacturer’s instructions. Cells were treated 48 h after transfection with stressor.

**Quantitative Real-time RT-PCR Analysis—**Following experimental treatments of cells, RNA was extracted and column-purified (Qiagen), and cDNA was generated by reverse transcription (Invitrogen). Quantitative real-time RT-PCR (qPCR) analysis was performed using Quantitect SYBR Green reagent (Qiagen) on a 7300 real-time PCR system (ABI). Relative gene expression was determined using the ∆ΔCT method, using β-actin as internal control. Primer specificity was confirmed by melting curve analysis. PCR primer sequences are provided in supplemental Table 1.

**Immunoblot Analysis—**Following experimental treatments, cells were washed with PBS and lysed in ice-cold radioimmu-
noprecipitation buffer, supplemented with protease inhibitors and phosphatase inhibitors (Roche Applied Science). Lysates were precleared by centrifugation, and then 20 μg of total protein was separated by SDS-PAGE electrophoresis (NuPAGE, Invitrogen) and transferred onto nitrocellulose (Hybond ECL, Amersham Biosciences). Subsequent immunoblotting and detection of enhanced chemiluminescence reaction by horse-radish peroxidase were performed by standard methods. Antibodies used were as follows: anti-CHOP (for human CHOP detection) and ATF4 from Genetex; anti-CHOP (for mouse CHOP detection) and β-actin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-SNAT2/SLC38A2 from AbCam; and anti-phospho-Thr-389 and total S6K1 and phospho-Ser-473 and total AKT antibodies from Cell Signaling. For immunoblot quantitation, chemiluminescence was captured with an Alpha Innotech Fluorchem Imager and analyzed with AlphaEaseFC software.

2-(Methylamino)isobutyric Acid (MeAIB) Uptake Assay—3T3L1 adipocytes cultured in 24-well plates were serum-starved for 16 h and then treated with various stressors in serum-free medium. For HEK293T, cells were seeded on poly-L-lysine 24-well plates and transfected with siRNAs. After 2 days, cells were serum-starved for 16 h and then treated with arsenite. Following stress treatments, cells were washed with Hepes-buffered saline (pH 7.7) and then incubated in 17 μM [14C]MeAIB (PerkinElmer Life Sciences), for 10 min at room temperature. Cells were then washed twice with cold Hepes-buffered saline and then lysed in 1% SDS, 50 mM sodium hydroxide. Equal protein quantities were mixed with scintillant, and radioactivity was measured. To determine specific System A uptake of [14C]MeAIB, the radioactive count measured from cells co-treated with competitive cold MeAIB (10 mM) was subtracted from all sample counts.

Statistical Analysis—Student’s t test with two tails was calculated where indicated in the figure legends. p ≤ 0.05 was considered significant. On bar charts, error bars indicate S.E. values. Statistics were calculated with GraphPad Prism software.

RESULTS

Genome-wide shRNA Screen Identifies Genes Required for Arsenite-induced ER Stress—We performed a FACS-based shRNA screen, assaying transcriptional activation of CHOP as a read-out of ER stress. CHOP, a member of the C/EBP family of transcription factors, is activated by PERK-ATF4, ATF6, and IRE1 (15, 32, 33) and thus receives positive inputs from all major UPR pathways. A transgene reporter encoding mCherry under control of the CHOP promoter was constructed (Fig. 1A) and stably introduced into HEK293 cells. This reporter demonstrated responsiveness to both arsenite and the classic ER stressor, tunicamycin (Fig. 1B). Induction of the reporter was dose-dependent, and treatment with 15 μM arsenite produced a ~10-fold increase in mean mCherry fluorescence and caused minimal cell toxicity (supplemental Fig. S1A).

A genome-wide lentiviral shRNA library, containing 65,000 shRNAs targeting ~20,000 human genes, was used in this study. The stable HEK293 reporter cells were transduced with the lentiviral shRNA library at a relatively low multiplicity of infection (0.3) to avoid multiple transductions per cell. The final cell population contained ~6 million independent viral integration events, providing ~90-fold coverage of the shRNA library. To identify genes required for arsenite-induced CHOP expression, reporter cells transduced with the shRNA library were first treated with arsenite (15 μM, 16 h) to activate the reporter. Cells with weak mCherry induction, presumably due to shRNA-mediated knockdown of CHOP “activators,” were isolated by FACS (Fig. 1C). Cells isolated by this procedure were regrown and subjected to further rounds of induction and FACS for enrichment of this “weak activator” cell population. shRNAs from this population were PCR-cloned and sequenced to identify target genes. D, primary screen results. Sequencing of 268 shRNA clones identified 168 annotated genes, which were selected for further analysis (for details, see “Results”). E, gene hits validated by shRNA and siRNA. Genes whose knockdown by shRNA and siRNA reduced arsenite-induced reporter activity or endogenous CHOP expression, respectively, were considered validated.

From this primary screen, we identified IRE1, which demonstrated that the screen was capable of identifying established ER stress modulators. The 17 primary screen hits were subjected to secondary screening, where the suppressive effect of individual
shRNAs on reporter induction was evaluated. Additionally, because of potential off-target shRNA effects, the ability of unique siRNAs to suppress endogenous CHOP induction in transfected HEK293T cells was examined. Four genes passed validation by these two methods and are listed in Fig. 1E and supplemental Fig. S1, B and C. Although the effect of single gene knockdowns on CHOP induction was modest, it was not unexpected because CHOP expression is controlled by multiple redundant UPR signaling pathways (15, 32, 33). Furthermore, the effect of siRNA and shRNA on target gene expression was redundant UPR signaling pathways (15, 32, 33). Furthermore, gene knockdowns on unexpected because 60% of control) (Fig. 2).

Among the validated gene hits, CDC37 is an Hsp90 co-chaperone that is essential for the maturation of elf-2α kinases HRI and GCN2 (34, 35). Indeed, Hsp90 is required for activation of IRE1, ATF6, and PERK branches of the UPR, as revealed by a recently developed specific Hsp90 inhibitor (36). MGAT2 is a Golgi-localized glycosyltransferase, which is up-regulated during plasma cell differentiation by XBP1 (37). PXDNL is a putative secreted peroxidase (38). SNAT2 is the most broadly expressed subtype of three System A transporters that mediate sodium-coupled uptake of small, aliphatic amino acids, such as glutamine, alanine, and cysteine (39).

Reduced SNAT2 Expression, Inhibition of Transporter Activity, or Glutamine Deprivation Suppresses Arsenite-induced ER Stress—There is a growing appreciation that cellular nutrient abundance is intimately tied to endoplasmic reticulum function (40). Because SNAT2 is an amino acid transporter that displays regulated expression (e.g. in response to amino acid deprivation and hyperosmotic stress (41, 42)), we further examined its role in arsenite-induced ER stress. Two different SNAT2 siRNAs suppressed arsenite-induced endogenous CHOP expression compared with control (~60% for siRNA 1, 80% for siRNA 2), as assessed by qPCR (Fig. 2A). This effect was achieved with ~60% (siRNA 1) or 45% (siRNA 2) SNAT2 knockdown efficiency. Induction of CHOP protein levels was also significantly suppressed with SNAT2 siRNA (~50% of control) (Fig. 2D). Interestingly, we observed ~3-fold induction of SNAT2 itself by arsenite, which could be inhibited by SNAT2 siRNAs (Fig. 2A). Importantly, we also observed that SNAT2 siRNAs could reduce the amount of spliced XBP1 induced by arsenite (~50% for siRNA 1, 67% for siRNA 2) (Fig. 2B). Splicing of XBP1 is another indicator of ER stress. Thus, the role of SNAT2 in arsenite response is not limited to CHOP induction but broadly affects the UPR. However, SNAT2 siRNAs do not have a significant suppressive effect on tunicamycin-induced CHOP (Fig. 2C), suggesting a specific role for SNAT2 in the arsenite response.

We next asked whether inhibition of SNAT2 transporter activity could suppress arsenite-induced ER stress. The amino acid analog MeAIB is a specific substrate of System A transporters that inhibits transporter activity at saturating concentrations (39). We examined the effect of MeAIB on arsenite-treated 3T3L1 adipocytes, which are commonly used in studies of ER stress due to their high metabolic activity. Treatment of adipocytes with 10 mM MeAIB for 3 h can reduce System A activity to ~30% (uptake assay; Fig. 3D). MeAIB suppressed CHOP induction by arsenite (~60% of control) (Fig. 2, E and G). Treatment with MeAIB also suppressed induction of GRP78 (~67% of control), another read-out of UPR activation (15) (Fig. 2F). We attribute the effect of MeAIB on 3T3L1 adipocytes to be mainly due to inhibition of SNAT2 because basal as well as arsenite-induced expression of other System A transporters is relatively low in this cell line (supplemental Fig. S2A).

Next, we determined whether the amino acids transported by SNAT2 mediate its effect on arsenic-induced ER stress. Glutamine is a SNAT2 substrate of particular importance because it is the most abundant free amino acid in plasma and in cell culture medium and is vital for cell metabolism and protein synthesis (43). Pretreatment with glutamine-free medium (3 h) suppressed arsenite induction of CHOP (~75% of control RNA, ~50% of control protein) and GRP78 (~62% of control) (Fig. 2, E–G). MeAIB treatment and glutamine starvation could not dampen significantly tunicamycin-induced CHOP or GRP78 (supplemental Fig. S2B). These data indicate that arsenite-induced ER stress and UPR require, at least in part, SNAT2-mediated amino acid transport.

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blocked arsenite-induced SNAT2 (Fig. 3F). This suggests that both oxidative stress and proteotoxic signals contribute to SNAT2 up-regulation by arsenite. ATF4 is a key transcription factor that coordinates amino acid metabolism during amino acid starvation, ER stress, and oxidative stress (17). Arsenite can induce eIF-2α phosphorylation, resulting in increased ATF4 protein levels (10, 11), and during amino acid starvation, ATF4 binds to an amino acid response element in the SNAT2 gene (46), suggesting a potential role in arsenite induction of SNAT2. Indeed, ATF4-targeting siRNA significantly suppressed arsenite-induced SNAT2 expression and System A activity (Fig. 3, G and H). Basal levels of SNAT2 expression and System A activity are, however, unchanged. Together, these data demonstrate that SNAT2 is part of an arsenite stress response and that up-regulation of SNAT2 by arsenite is dependent on the stress-responsive transcription factor ATF4.

**mTOR Activation Links SNAT2 Activity and Arsenite-induced ER Stress**—The data presented so far indicate that up-regulation of SNAT2 expression and transporter activity contribute to arsenite-induced ER stress. How might this occur? Activation of mTOR signaling is highly responsive to intracellular amino acid levels (47). A critical role for glutamine as a limiting nutrient in mTOR activation has recently been described (48), and indeed, depletion of intracellular glutamine through inhibition of SNAT2 dampens mTOR activity in muscle cells (49). mTOR is a key regulator of cell growth and promotes protein synthesis (50). An overabundance of nutrients and elevated mTOR signaling can cause ER stress (22, 51).
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Thus, we hypothesized that arsenite might contribute to ER stress through SNAT2 induction, leading to enhanced mTOR signaling.

As a read-out of mTOR signaling, we examined the phosphorylation of S6K1 on Thr-389. S6K1 is a direct substrate of the mTOR kinase within mTOR complex 1 (mTORC1). Cells were serum-starved overnight to reduce basal mTOR activity and then treated with arsenite. Arsenite increased S6K1 phosphorylation in a dose- and time-dependent manner in both 3T3L1 adipocytes and HEK293T cells (Fig. 4, A and B, and supplemental Fig. S3, A and B). Increases in S6K1 phosphorylation were apparent from 1.5 to 6 h of arsenite treatment (50 μM), and from 2 to 50 μM (6 h). Importantly, the latency, response time, and dose response of S6K1 phosphorylation are coincident with System A induction (Fig. 3, D and E). Increased S6K1 phosphorylation was completely blocked by rapamycin, confirming that this reflects increased mTOR signaling. However, arsenite did not stimulate AKT phosphorylation, indicating that mTOR activation by arsenite does not involve this upstream pathway.

We next examined whether increased mTOR activity contributes to arsenite-induced ER stress. Inhibition of mTORC1 by rapamycin suppressed arsenite-induced CHOP expression in 3T3L1 adipocytes (~10% compared with control) (Fig. 4C). Rapamycin treatments in HEK293T cells also reduced CHOP induction (~50% of control) and XBP1 splicing (~60% of control) (Fig. 4D and supplemental Fig. S3C). Therefore, mTOR activity is activated by arsenite and contributes to arsenite-induced ER stress.

To test whether SNAT2 contributes to mTOR activation by arsenite, siRNA knockdown of SNAT2 was performed. Compared with non-targeting control, SNAT2-targeting siRNA reduced phosphorylated S6K1 levels induced by arsenite (~60% of control) (Fig. 4E). The effect of SNAT2 inhibition was also tested. MeAIB co-treatments significantly reduced arsenite-induced S6K1 phosphorylation (Fig. 4F). We attribute the effect of MeAIB mainly to inhibition of SNAT2 and possibly SNAT1, whose expression is abundant and modestly inducible by arsenite in HEK293T cells (supplemental Fig. S3D). Glutamine starvation also blunted arsenite-stimulated S6K1 phosphorylation (Fig. 4F), indicating the importance of glutamine availability.

System L transporters couple the efflux of intracellular glutamine to the import of large branched amino acids, which are the most potent mTOR activation amino acids (48, 52, 53).
System A transporters, such as SNAT2, therefore, directly supply amino acids that drive System L function. LAT1/SLC7A5, a transporter that exchanges glutamine for leucine, can be inhibited by 2-amino-bicyclo-(2,2,1)-heptanonecarboxylic acid (BCH), a specific System L inhibitor (54). BCH alone inhibited S6K1 phosphorylation and blunted the arsenite response (Fig. 4F). Furthermore, BCH could significantly suppress CHOP expression and XBP1 splicing induced by arsenite (Fig. 4, C and D, and supplemental Fig. S3C). Together, these data suggest that arsenite-induced ER stress and activation of the UPR are enhanced by SNAT2- and LAT1-mediated mTOR activation (Fig. 4G).

**DISCUSSION**

In this study, we performed a genome-wide functional RNAi screen to identify genes required for the ER stress response induced by arsenic. The data presented demonstrate, to our knowledge, the first utilization of a functional RNAi screen for ER stress activation in mammalian cells. The data also highlight the utility of RNAi screening to elucidate cellular mechanisms of environmental toxins. The screen identified several putative modulators of arsenite-induced ER stress, including genes with known functions in stress response (CDC37, SNAT2). Mechanistic studies of SNAT2 established an important role for this neutral amino acid transporter in linking arsenite exposure, mTOR activation, and ER stress.

Our findings identify SNAT2 as a novel component of the arsenite stress response. We demonstrated that SNAT2 and its transporter activity are required for arsenite-induced ER stress, as measured by induced CHOP, XBP1 splicing, and GRP78. Furthermore, SNAT2 expression and activity are induced by arsenite. SNAT2 is the most broadly expressed subtype of three System A transporters that mediate sodium-coupled uptake of small, aliphatic amino acids, such as glutamine, alanine, and cysteine (39). Regulation of SNAT2 expression by hyperosmotic stress and amino acid deprivation has previously been documented (41, 42), but this is the first demonstration of SNAT2 regulation by arsenite. We further demonstrate that arsenite induction of SNAT2 requires ATF4 and both oxidative stress and proteotoxic upstream signals. During amino acid deprivation, SNAT2 expression can be regulated by both transcriptional activation, via ATF4 (46), and cap-independent translation of the mRNA allowed by the IRES in the 5’-UTR (44). Similar regulatory mechanisms are likely to be activated by arsenite, which can induce eIF-2α phosphorylation and ATF4 expression (10, 11). During stress conditions, ATF4-mediated induction of amino acid transporters, such as LAT1 (17) and SNAT2, may support the increased demand for protein chaperone synthesis as well as the supply of proteins and peptides involved in redox reactions (i.e. glutathione), therefore mitigating oxidative stress (43). Glutamine also supports glucose
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metabolism (43), which is severely compromised during arsenite stress (55). Thus, several potential adaptive mechanisms are supported by SNAT2. SNAT2 activity cannot be induced by tunicamycin or thapsigargin, indicating its specificity in the arsenite response. Previous studies have shown that thapsigargin can suppress SNAT2 expression by an unknown mechanism downstream of ATF4 recruitment (56), suggesting that ER stress may generate a repressive signal. Thus, one possible explanation for the apparent specificity in SNAT2 induction is that arsenite does not elicit an adequate repressive ER stress signal in contrast to potent ER stressors tunicamycin and thapsigargin.

Previous studies have demonstrated that arsenite can activate mTOR (57, 58), which we also observe in HEK293T and 3T3L1 adipocytes. Multiple stresses impinge on mTOR, which maintains the appropriate balance between anabolic processes, like protein synthesis, and catabolic processes, such as autophagy. It is not clear how arsenite activates mTOR, although very recently Raptor phosphorylation by p38β has been suggested as one potential mechanism (59). Intracellular amino acid availability is recognized as a major mTORC1 regulatory mechanism (47), and recent studies have revealed an important regulatory role for glutamine transporters (48, 49, 52). Cellular import of glutamine can be regulated by at least four different transporter types (Systems A, N, ASC, and L) (60). SLC1A5 (a System ASC transporter) and SNAT2 have been shown to regulate basal mTORC1 activity in HeLa and muscle cells, respectively (48, 49, 52). By concentrating intracellular amino acids like glutamine, these transporters provide exchange substrates for System L transporters (LAT1) and therefore drive the import of branched amino acids like leucine, which are potent mTORC1 activators (48, 52). Our findings suggest that during arsenite stress, amino acid signaling to mTORC1 can be enhanced by elevated SNAT2 transporter activity. First, there is a temporal correlation in S6K1 phosphorylation and SNAT2 expression and System A activity. Furthermore, specific inhibition of System A, glutamine starvation, or SNAT2-targeting siRNA suppressed S6K1 phosphorylation. Last, inhibition of LAT1 also suppressed arsenite-induced S6K1 activation. In addition, consistent with an amino acid signaling mechanism, the PI3K/AKT pathway is not activated. These findings indicate that SNAT2 transporter activity is important for mTOR activation during arsenite stress.

During arsenite stress, SNAT2 up-regulation may promote adaptation through mTOR-dependent as well as mTOR-independent anabolic processes, as described above. However, this can lead to overloading of the ER and consequently ER stress and provides an explanation as to why inhibition or reduced expression of SNAT2 and LAT1 suppressed arsenite-induced ER stress. Previous studies have demonstrated that overactive mTORC1 activity can cause ER stress and activate the UPR (51), presumably due to increased protein synthesis in the ER. Consistent with this view, inhibition of mTORC1 with rapamycin in 3T3L1 adipocytes and HEK293T cells partially blocked arsenite-induced ER stress. Together, our data support a model where arsenite activation of SNAT2, in an ATF4-dependent manner, increases System A activity, which drives glutamine import. This leads to increased leucine import and mTORC1 activity and eventually ER stress (Fig. 4G).

Environmental arsenic exposure in human populations has been linked to type 2 diabetes and cancer (1, 2), but the underlying mechanisms are poorly understood. Our study suggests that altered mTOR signaling by SNAT2 activation may play an important role. A better understanding of SNAT2 activity under arsenic stress conditions and its role in arsenic-related diseases will require physiological animal models. Because ER stress and JNK activation play major roles in the negative feedback on insulin action and glucose metabolism (22, 51), our findings suggest that a SNAT2-mTOR-ER stress mechanism may contribute to arsenic’s inhibitory effects on insulin signaling (55). Already, a role for SNAT2 in “nutritional stress” has been suggested because its expression and activity in the liver of diabetic mice is elevated (61, 62). Induction of SNAT2 activity may also play a role in arsenic-related cancers, through elevation of mTOR activity and UPR activation, which can promote tumor survival (24, 25, 63).

In addition to SNAT2, we have identified several other putative regulators of arsenic-induced ER stress response. The hits we report here represent only a partial list of new genes involved. Indeed, we note that the recovery of a relatively small number of shRNAs (268) from our screen limited our depth of discovery. Investigations that utilize “deep sequencing” technology will likely uncover additional potential modulators of arsenic-induced ER stress. Functional characterization of these genes and the already identified and validated RNAi hits will reveal more novel insights into the mechanisms involved and could inform human epidemiologic approaches. For example, guided by this gene set, epidemiologic and genetic studies may uncover relevant gene variants associated with the arsenic-related diabetogenic or carcinogenic effect in humans. Furthermore, because the ER stress response is a critical component of general environmental surveillance in cells, the molecular mechanisms uncovered by our screen may be relevant to other environmental exposures that cause ER and oxidative stress.

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