Involvement of c-Met- and Phosphatidylinositol 3-Kinase Dependent Pathways in Arsenite-Induced Downregulation of Catalase in Hepatoma Cells

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Catalase protects cells from reactive oxygen species-induced damage by catalyzing the breakdown of hydrogen peroxide to oxygen and water. Arsenite decreases catalase activity; it activates phosphatidylinositol 3-kinase (PI3K) and its key downstream effector Akt in a variety of cells. The PI3K pathway is known to inhibit catalase expression. c-Met, an upstream regulator of PI3K and Akt, is also involved in the regulation of catalase expression. To examine the involvement of c-Met and PI3K pathways in the arsenite-induced downregulation of catalase, catalase mRNA and protein expression were analyzed in the human hepatoma cell line HepG2 treated with arsenite and either an inhibitor of c-Met (PHA665752 (PHA)) or of PI3K (LY294002 (LY)). Arsenite treatment markedly activated Akt and decreased the levels of both catalase mRNA and protein. Both PHA and LY attenuated arsenite-induced activation of Akt. PHA and LY treatment also prevented the inhibitory effect of arsenite on catalase protein expression but did not affect the level of catalase mRNA. These findings suggest that arsenite-induced inhibition of catalase expression is regulated at the mRNA and post-transcriptional levels in HepG2 cells, and that the post-transcriptional regulation is mediated via c-Met- and PI3K-dependent mechanisms.

Key words catalase; arsenite; c-Met; phosphatidylinositol 3-kinase; Akt

Cellular defense systems, including a variety of antioxidant molecules and enzymes such as superoxide dismutase, glutathione peroxidase and catalase, ensure that reactive oxygen species (ROS) are maintained at relatively low levels in normal conditions.1,2 Arsenic and its compounds are well-documented environmental toxicants3 that induce oxidative damage by increasing the production of ROS. Oxidative damage has been suggested to play a key role in arsenic-induced toxicity.3–7

Catalase protects cells from ROS-induced damage by catalyzing the breakdown of hydrogen peroxide (H2O2) into oxygen and water. Arsenic has been demonstrated to decrease catalase expression both in animal tissues and cultured cells. Catalase activity is significantly decreased in the liver of rats treated with arsenite.8–10 Arsenite treatment in vitro decreases catalase activity in human fibroblasts.11 In a human keratinocyte cell line, it was also shown to decrease levels of catalase mRNA and protein.11

Phosphatidylinositol 3-kinases (PI3Ks) are key components for the activation of Akt signaling. In the PI3K/Akt pathway, formation of 3-phosphoinositides by PI3K enables the activation of Akt by phosphoinositide-dependent protein kinases 1 and 2, which phosphorylate Akt at threonine residue 308 (Thr308) and serine residue 473 (Ser473), respectively. PI3K/Akt is thought to play a pivotal role in regulating cell proliferation, survival, metabolism and cancer progression. The PI3K/Akt pathway is downstream of c-Met receptor tyrosine kinase, a receptor for hepatocyte growth factor.12–14 In c-Met-deficient mouse hepatocytes, the level of catalase protein is constitutively high,15 suggesting that a c-Met-dependent pathway is involved in catalase expression. Rat liver epithelial cells transformed by chronic exposure to arsenite show upregulated c-Met expression.16

Some studies indicate that arsenite induces the activation of PI3K/Akt. In human keratinocytes, for example, it induces the phosphorylation of Akt at both Ser473 and Thr308, which is inhibited by treatment with PI3K inhibitors such as Wortmannin and LY294002 (LY).17 Arsenite-induced activation of both PI3K and Akt and the attenuation of arsenite-induced activation of Akt by PI3K inhibitors have also been demonstrated in human prostate carcinoma cells18 and mouse epidermal cells.19

PI3K/Akt plays an important role in regulating catalase expression. Venkatesan et al. examined the role of PI3K/Akt in H2O2-induced downregulation of catalase in mesangial cells.20 They found that LY significantly attenuates the inhibitory effect of H2O2 on the level of catalase protein and that the expression of the tumor suppressor protein phosphatase and tensin homolog (PTEN), a modulator of PI3K pathway,21,22 increases catalase protein levels. It is the expression of dominant negative Akt that significantly activates the catalase gene promoter and prevents the inhibitory effect of H2O2 on the catalase protein level.

Previous studies have demonstrated arsenite-induced activation of PI3K and c-Met- and PI3K-dependent inhibition of catalase expression. The aim of this study was to examine the involvement of c-Met- and PI3K-dependent pathways in arsenite-induced inhibition of catalase expression. Catalase mRNA and protein expression have been analyzed in human hepatoma cells treated with sodium arsenite and an inhibitor of either c-Met or PI3K.

MATERIALS AND METHODS

Chemicals All chemicals used were of reagent grade or higher and were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), unless otherwise specified.

Cell Treatment Human hepatoma cell line HepG2 was
grown in RPMI 1640 medium with 2 mg/ml sodium bicarbonate, 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a 5% CO₂ incubator. Twenty-four hours after the cells were plated, the culture medium was removed and replaced with medium containing various concentrations of sodium arsenite. Solutions of c-Met inhibitor PHA665752 (PHA, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and PI3K inhibitor LY (BioVision, Mountain View, CA, U.S.A.) dissolved in dimethylsulfoxide (DMSO) were added to the culture plates. The final concentration of DMSO in both control and test cultures did not exceed 0.05%. For the c-Met and PI3K inhibition studies, HepG2 cells were treated with PHA for 4 h followed by sodium arsenite for 24 h, and co-treated with LY and sodium arsenite for 24 h, respectively.

Cell Viability 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction was used to assess cell viability. Briefly, 10× MTT stock solution (5 mg/ml in phosphate buffered saline, pH 7.4) was added to each culture well, and the plates were incubated at 37 °C for 4 h. The MTT solution was then aspirated and 0.2 ml of acidified phosphate buffered saline, pH 7.4) was added to each culture, the plates were analyzed using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at a wavelength of 560 nm.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from HepG2 cells using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. The amount of RNA in each sample was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and 1 μg of total RNA was reverse transcribed using reverse transcriptase and random hexamers (Promega, Madison, WI, U.S.A.). Real-time RT-PCR was performed with cDNAs and gene-specific primers (Table 1) using ABI SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.) in an ABI Prism 7000 thermocycler (Applied Biosystems). Samples were first denatured for 10 min at 95 °C and then subjected to forty cycles of amplification and quantification (15 s at 95 °C, 1 min at 60 °C), followed by a melting curve program (60—99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement).

Western Blot Total proteins were extracted from HepG2 cells with a Pro-Prep protein extraction kit (Intron, Kyungkido, Korea) and protein concentration was determined with a Pro-Measure kit (Intron) according to the manufacturer’s instructions. Samples containing 10 μg of protein were denatured by boiling for 10 min in 5× sodium dodecyl sulfate (SDS) gel loading buffer (5% SDS; 0.225 M Tris, pH 6.8; 50% glycerol; 0.05% bromophenol blue; 0.25 mM dithiothreitol), separated by SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. The membranes were incubated with mouse anti-catalase antibody (ab16731, Abcam, Cambridge, MA, U.S.A.), rabbit anti-Akt antibody (#9272, Cell Signaling Technology, Beverly, MA, U.S.A.), rabbit anti-phospho-Akt (Ser473) antibody (#9271, Cell Signaling Technology), rabbit anti-phospho-Akt (Thr308) antibody (#4065, Cell Signaling Technology) and mouse anti-β-actin antibody (A5441), followed by incubation with horse-radish peroxidase (HRP)-conjugated sheep anti-mouse immunoglobulin G (IgG) (NA931, Amersham Biosciences, Arlington Heights, IL, U.S.A.) or HRP-conjugated donkey anti-rabbit IgG (NA934, Amersham Biosciences) as secondary antibody. Blots were developed using a chemiluminescence detection reagent kit. The Western blot procedure provides a qualitative, not quantitative, comparison between samples; therefore, no statistical analysis was performed.

Statistical Analysis All data are expressed as mean ± standard deviation (S.D.), and the non-parametric Mann–Whitney U test was used to compare selected pairs of groups. An IC₅₀ value was determined by probit analysis. All statistical analyses were performed using SPSS 18 software (SPSS, Chicago, IL, U.S.A.). A p-value of less than 0.05 was considered significant.

RESULTS

Cytotoxicity To assess the cytotoxic effects of arsenite on HepG2 cells, the MTT assay was used as a measure of cell viability. Cells were incubated for 24 h with different doses of arsenite ranging from 2.5 μM up to 80 μM (Fig. 1). Cell viability significantly decreased in a dose-dependent manner after a 24 h treatment with arsenite doses of 20 μM or higher, and the IC₅₀ (the dose required for 50% reduction in cell viability) of arsenite was calculated to be 27.5 ± 2.40 μM. In all subsequent experiments, the highest concentration of arsenite used to treat cells was 15 μM, approximately half the IC₅₀.

Inhibition of Catalase Expression Next, the expression
of catalase mRNA and protein in arsenite-treated HepG2 cells was investigated. Arsenite treatment decreased catalase mRNA levels in a dose-dependent manner (Fig. 2A). Catalase mRNA expression was decreased 1.7-, 1.8- and 3.4-fold compared to untreated controls (UC) after treatment with arsenite at 3.75, 7.5 and 15 μM, respectively. Compared with UC, arsenite also decreased the level of catalase protein in the cells in a dose-dependent manner (Fig. 2B).

Expression of MT1b and HO-1 mRNA
To study the effect of arsenite on the expression of oxidative stress-responsive gene products, levels of metallothionein 1b (MT1b) and heme oxygenase-1 (HO-1) mRNA in arsenite-treated cells were analyzed. Like catalase, both MT1b and HO-1 play an important role in scavenging cellular ROS. Consistent with previous studies, the mRNA levels of MT1b and HO-1 were increased significantly compared to UC after 24 h treatment with arsenite doses of 3.75 μM or higher (Fig. 3). Thus, whereas catalase expression is inhibited in arsenite-treated cells (Fig. 2), expression of the functionally-related genes, MT1b and HO-1, is up-regulated.

Activation of Akt
Previous studies have demonstrated arsenite-induced activation of Akt in a variety of cells. To determine whether Akt is activated in HepG2 cells after treatment with arsenite, phosphorylation of Akt at Ser473 and Thr308 was examined. Arsenite markedly induced Akt phosphorylation of both residues in a dose-dependent manner, while the level of total Akt protein remained unaffected (Fig. 4).

Effect of a c-Met Inhibitor
Since c-Met deficiency has been shown to increase the level of catalase protein in mouse hepatocytes, the effect of a c-Met inhibitor PHA on catalase regulation in arsenite-treated cells was investigated (Fig. 5). The level of Akt protein in the cells was unaffected by treatment with PHA alone. PHA, however, blocked arsenite-induced Akt phosphorylation at Ser473 and Thr308 and attenuated the inhibitory effect of arsenite on catalase protein level in a dose-dependent manner (Fig. 5A). These findings
indicate that a c-Met pathway is responsible for the reduced expression of catalase protein in arsenite-treated cells. PHA treatment also increased the catalase protein level in cells that were not treated with arsenite.

To determine whether attenuation by PHA of arsenite-induced catalase protein expression inhibition results from a change in mRNA levels, real-time RT-PCR was performed (Fig. 5B). In contrast to the inhibitory effect of PHA on catalase protein levels (Fig. 5A), it did not affect the level of catalase mRNA in arsenite-treated and -untreated cells. These findings indicate that c-Met is not involved in regulating catalase mRNA expression in HepG2 cells.

Effect of PI3K Inhibition

The PI3K inhibitor LY had a similar effect on catalase expression in HepG2 cells to PHA (Fig. 6). LY treatment attenuated arsenite-induced Akt phosphorylation at Ser473 and Thr308 in a dose-dependent manner, while the Akt protein level was not affected (Fig. 6A). The catalase protein level increased after LY treatment in arsenite-treated cells, suggesting that inactivation of PI3K attenuates the arsenite-induced decrease in catalase protein levels. LY treatment also increased the level of catalase protein in the cells that were not treated with arsenite.

To determine whether the change in the level of catalase was a result of a change in the level of catalase mRNA, catalase real-time RT-PCR was carried out on cells treated with LY and arsenite (Fig. 6B). In contrast to the inhibitory effect of LY on catalase protein levels, LY, like PHA, did not block arsenite-induced inhibition of catalase mRNA expression in the cells (Fig. 5). These findings suggest that PI3K is not involved in arsenite-induced inhibition of catalase mRNA expression in HepG2 cells.

DISCUSSION

The aim of this study was to investigate the involvement of c-Met- and PI3K-dependent pathways in arsenite-induced inhibition of catalase expression. In agreement with other studies of cultured cells and animal tissues, arsenite-induced inhibition of catalase expression was demonstrated. Interestingly, a decrease in the gene expression of catalase, a major ROS-scavenging enzyme, occurred in cells where oxidative stress-responsive gene products such as MT1b and HO-1 were highly up-regulated. Based upon the catalytic characteristics of catalase, reduced catalase activity in cells undergoing oxidative stress is likely to predispose those cells to H₂O₂-induced toxicity.

How catalase expression is regulated during oxidative stress is unclear. Catalase activity, protein and/or mRNA levels have been found to be increased, unchanged or decreased during oxidative stress. A ROS-mediated, biphasic model of catalase regulation has, however, been proposed in which catalase is stimulated by c-Abl/Arg-mediated
phosphorylation at lower ROS levels, while in the event of uncontrollable ROS levels, catalase is degraded by ubiquitin-mediated proteasomal proteolysis, which thereby induces cell death. It has also been shown that treatment with ceramides, which induce oxidative damage, results in proteolytic cleavage of catalase by activated caspase-3. Since arsenite-treated cells are in a state of high oxidative stress, as indicated by the marked induction of MT1b and HO-1 expression, it is possible that catalase is subject to proteolytic degradation in these cells.

This study has provided the first evidence for the involvement of c-Met and PI3K pathways in the arsenite-induced inhibition of catalase expression. Inactivation of c-Met and PI3K led to increased levels of catalase protein in both arsenite-treated and -untreated cells, with no effect on the level of catalase mRNA. These findings suggest that the primary effect of c-Met and PI3K inactivation on catalase levels in HepG2 cells occurs post-transcriptionally. Since c-Met and PI3K inhibitors both affected the arsenite-induced inhibition of catalase expression similarly, it is likely that a c-Met/PI3K/Akt pathway is involved in post-transcriptional regulation of catalase in arsenite-treated HepG2 cells.

Venkatesan et al. reported that ROS treatment inhibits the transcription of catalase in mesangial cells. The arsenite-induced decrease in catalase mRNA found in the present study could plausibly be the result of transcriptional inhibition. However, in the same report, dominant negative Akt was shown to increase catalase mRNA in mesangial cells; this is inconsistent with the observation presented here that Akt inactivation did not enhance the levels of catalase mRNA. The specific mechanisms underlying this apparent discrepancy remain to be elucidated.

In summary, the findings of the present study suggest that arsenite-induced inhibition of catalase expression is regulated at the mRNA and post-transcriptional levels in hepatoma cells, and that post-transcriptional regulation of catalase is mediated via c-Met- and PI3K-dependent mechanisms.

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