Developing Biomarkers of Arsenic Exposure and Toxicity

Project Scope

Arsenic is a highly toxic element that occurs naturally in the environment and is also released by human activities. The most important human exposure source is contaminated drinking water. An important toxic mechanism of inorganic arsenic (iAs) is the interaction of trivalent iAs, (arsenite or iAs^{III}), with thiol-containing residues of peptides and proteins. (See also Exhibit 1 for a list of arsenic-related acronyms used in this report.) This interaction can result in the inhibition of a number of enzymes and the inactivation of critical cellular receptors. Glutathione reductase (GR) has been identified as a key enzyme that is sensitive to inhibition by iAs^{III}. GR activity regulates the intracellular ratio of glutathione (GSH, a major intracellular antioxidant) to glutathione disulfide (GSSG); the GSH:GSSG ratio is an important indicator of cellular redox status. Changes in the cellular redox status have been linked to modulations in gene transcription that underlie uncontrolled cell proliferation, an early step in the development of cancer.

Methylated arsenicals (organic arsenic compounds) that contain trivalent arsenic (As^{III}) are orders of magnitude more potent GR inhibitors than iAs^{III}. Thus, production of methylated trivalent arsenicals in the course of iAs metabolism may cause GR inhibition, shifting the GSH:GSSG ratio and the cellular redox balance. Thioredoxin reductase (TR) is another important cellular enzyme that regulates the response of cells to oxidative stress through reduction of thioredoxin (TRx).

The objectives of this research were to:

1. Characterize the effects of arsenicals on GR activity, GSH:GSSG ratio, and the production of reactive oxygen species (ROS) in cultured human cells and compare these effects with patterns of arsenic metabolism and toxicity using the same cells;

Grant Title and Principal Investigators

Arsenicals, Glutathione Reductase and Cellular Redox Status (EPA Grant #R826136)

Miroslav Styblo, Melinda A. Beck, Luz M. Del Razo, Shan Lin, Felecia Walton, University of North Carolina, Chapel Hill; William R. Cullen, University of British Columbia

Key Findings and Implications

Analytical Accomplishments:

- Methylated trivalent arsenicals, particularly methylarsonous acid derivatives, are much more toxic in cultured human and animal cells than arsenite.
- Glutathione reductase activity and glutathione:glutathione disulfide ratios are not sensitive markers of arsenic toxicity at low exposure levels (below cytotoxic levels).
- Thioredoxin reductase (TR) activity may be a sensitive marker of methylarsonous acid toxicity at low exposure levels (below cytotoxic levels).
- Methylarsonous acid or dimethylarsinous derivatives were found in culture medium, indicating that these toxic metabolites could be released from hepatic cells and translocated via blood circulation to nonmethylating tissues or cells.

Implications of Research:

- Urine analysis of humans chronically exposed to inorganic arsenic indicates that trivalent methylated arsenicals—methylarsonous acid and dimethylarsinous derivatives—are natural products of the metabolism of inorganic arsenic in humans. Therefore, urinary levels of these two compounds may be used as indicators of exposure to inorganic arsenic.
- Trivalent arsenicals induce reactive oxygen species production in cultured mammalian cells at low exposure levels, and TR is a sensitive biomarker for trivalent arsenicals toxicity. These effects, which are not associated with acute toxicity, may be early indicators of arsenic efffects.

Publications include 10 peer reviewed journal articles, 3 book chapters, and 26 conference/workshop presentations.

Project Period: September 1997 to August 2000

Relevance to ORD's *Drinking Water Research Multi-Year Plan* (2003 Edition)

This project contributes directly to the first of three Long-term Goals for drinking water research: (1) by 2010, develop scientifically sound data and approaches to assess and manage risks to human health posed by exposure to regulated waterborne pathogens and chemicals, including those addressed by the Arsenic, M/DPB, and Six-Year Review Rules.

This research provides information regarding the use of early indicators of toxicity and exposure to arsenic to help assess and manage public health risks resulting from arsenic exposure. It shows that urinary levels of methylarsonous acid (MAs^{III}) and dimethylarsinous acid ($DMAs^{III}$) may be used as indicators of exposure to arsenic from contaminated drinking water, and that reactive oxygen species production in cells is an early indicator of arsenic exposure at concentrations below acutely toxic levels. Metabolism and mechanistic data generated by the grant will improve dose-response assessments for arsenic.

2. Characterize interactions of iAs and methylated arsenicals with GR purified from human cells; and
3. Characterize GR activity and GSH:GSSG ratio in tissues of laboratory animals exposed *in vivo* to arsenicals.

The overall goal of this research was to establish whether GR activity and the GSH:GSSG ratio can serve as sensitive markers of arsenic toxicity in individuals exposed to iAs from the environment. The original objectives were modified as the research proceeded, to reflect new findings and to focus on potentially more sensitive markers of arsenic toxicity in human cells, such as TR activity.

Exhibit 1
Arsenic-Related Acronyms

As - arsenic
iAs - inorganic arsenic
iAs^{III} - trivalent inorganic arsenic, also called arsenite
iAs^{V} - pentavalent inorganic arsenicals, also called arsenate
MAs - methyl arsenic (encompasses all this group)
  MAs^{III} - methylarsonous acid
  $MAs^{III}O$ - methylarsine oxide
  $MAs^{III}I_2$ - methylarsonous diiodide
DMAs - dimethyl arsenicals (encompasses all this group)
  $DMAs^{III}$ - dimethylarsinous acid
  $DMAs^{III}I$ - dimethylarsinous iodide
$DMAs^{III}GS$ - dimethylarsinous glutathione complex

Project Results and Implications

Metabolism and Toxicity of Arsenicals in Cultured Cells: The investigators measured inorganic toxicity in four cultured human cell lines derived from tissues that are targets for carcinogenic effects of iAs—primary hepatocytes (liver), normal epidermal keratinocytes (NHEK; skin), bronchial epithelial cells (HBEC; lung), and UROtsa (bladder epithelial cells). In these cell lines, trivalent arsenicals were significantly more cytotoxic than their pentavalent counterparts. Regardless of the cell type, trivalent monomethylated arsenicals, methylarsine oxide ($MAs^{III}O$) and methylarsonous diiodide ($MAs^{III}I_2$), were found to be the most potent cytotoxins, with LC_{50} values (the concentration that resulted in a 50 percent decrease in cell viability within 24 hours) ranging from 0.8 to 5.5 µM. The dimethylarsinous acid ($DMAs^{III}$) derivatives, dimethylarsinous glutathione complex ($DMAs^{III}GS$) and dimethylarsinous iodide ($DMAs^{III}I$), were as cytotoxic as methylarsonous acid (MAs^{III}) species, and more cytotoxic than iAs^{III} in some cell types. Similar cytotoxic patterns of cytoxicity were found in primary rat and primary guinea pig hepatocytes used throughout the study as positive and negative metabolic controls, respectively. The methylation rates for iAs^{III} in the various types were also quantified. Primary rat hepatocytes were shown to be superior methylators of iAs^{III}, while primary human hepatocytes were less active. The other cell types showed little or no methylation capacity for iAs^{III}. The researchers found no apparent correlation between the capacity of cells to methylate iAs and their sensitivity to the cytotoxic effects of trivalent arsenicals.

Effects of Arsenicals on GR Activity and Redox Status in Cultured Cells: Short-term exposures to cytotoxic concentrations of iAs^{III} or $MAs^{III}O$ resulted in a concentration-dependent inhibition of GR activity, and decreased GSH concentrations in cultured cells. No changes in GSSG concentration were detected. The GSH:GSSG ratio, a marker of the cellular redox status, decreased significantly in exposed cells as a result of GSH depletion. $MAs^{III}O$ was considerably more potent than iAs^{III} inhibiting GR and decreasing GSH concentration. Exposures to either arsenical did not change activity of glutathione peroxidase (GPx), another enzyme involved in GSH turnover in the cell. Similar effects of iAs^{III} and $MAs^{III}O$ on GR activity and GSH concentration were observed in primary human hepatocytes, NHEK, and UROtsa cells. In all cell types tested, the inhibition of GR activity and/or depletion of GSH were associated only with exposures to cytotoxic concentrations of iAs^{III} and $MAs^{III}O$. These data suggest that GR activity and GSH:GSSG ratio are not sensitive markers of arsenic toxicity at low exposure levels. $DMAs^{III}$ derivatives (up to 10 µM) did not modify GR activity or GSH concentrations in cultured cells. Exposures to iAs^{III} or MAs^{III} were associated with an increased production of peroxides as monitored in intact cells as measured by dichlorofluorescein fluorescence. The increased peroxide levels were detected in cells exposed to both high (cytotoxic) and to low (noncytotoxic) concentrations of iAs^{III} or MAs^{III}. Thus, induction of ROS in human cells may be an early sign of the toxicity of trivalent arsenicals.

Effects of Trivalent Arsenicals on Thioredoxin Reductase Activity in Cultured Cells: Unlike GR activity, the activity of TR was very sensitive to inhibition by trivalent arsenicals, particularly by MAs^{III} species. In primary rat and human hepatocytes, exposure to 1 µM $MAs^{III}O$ inhibited TR activity by about 30 percent. Fifty times higher concentration of iAs^{III} was required to attain a comparable degree of inhibition. In methylating rat hepatocytes exposed to iAs^{III}, TR inhibition correlated with production and intracellular concentration of MAs, but not iAs, indicating that trivalent form of MAs, MAs^{III}, accumulated in cells and was responsible for the inhibitory effect. These data suggest that inhibition of TR activity may be a sensitive marker of the production and accumulation of MAs^{III} in tissues of individuals exposed to iAs. Because TR activity affects a range of important biochemical functions (e.g., redox metabolism of TRx and ascorbate, redox sensitive mechanisms involved in the signal transduction pathway), inhibition of TR by MAs^{III} may be a very important mechanism contributing to the adverse effects associated with exposures to this arsenical.

Analysis of Methylated Trivalent Metabolites in Biological Samples: The researchers optimized a hydride generation atomic absorption spectrophotometrical (HG-AAS) technique for analysis of oxidation states of methylated metabolites in biological matrices to provide direct information about formation of MAs^{III} in the course of iAs metabolism in humans. Using this technique, methylated trivalent arsenicals were analyzed in cultured human cells exposed to iAs and in urine from individuals chronically exposed to iAs via contaminated drinking water. Human hepatocellular carcinoma (HepG2) cells exposed to iAs^{III} (0.1, 1, or 10 µM) synthesized $DMAs^{III}$ at all exposure levels. In contrast, MAs^{III} was detected only in cultures exposed to 1 or 10 µM iAs^{III}. Notably, significant amounts of MAs^{III} or $DMAs^{III}$ were found in culture medium, indicating that these toxic metabolites can be released *in vivo* from hepatic cells and translocated via blood circulation to nonmethylating tissues or cells. This indicates that the toxic metabolites may reach sensitive tissues even if those sensitive tissues do not have the ability to produce the toxic metabolites. Both MAs^{III} or $DMAs^{III}$ were found in urine samples collected from residents of Zimapan region (Mexico) who drink water contaminated with iAs ranging from 30 to 1,100 µg per liter). In urine from these individuals, MAs^{III} accounted for up to 9 percent of the total urinary MAs; $DMAs^{III}$ accounted for up to 31 percent of the total urinary DMAs. The amounts of MAs^{III} and $DMAs^{III}$ in urine positively correlated with total urinary arsenic. These results show that toxic trivalent methylated arsenicals, MAs^{III} and $DMAs^{III}$, are natural products of the metabolism of iAs in humans.

Investigators

M. Styblo, University of North Carolina, Chapel Hill
M.A. Beck, University of North Carolina, Chapel Hill
W.R. Cullen, University of British Columbia
L.M. Del Razo, University of North Carolina, Chapel Hill
S. Lin, F. Walton, University of North Carolina, Chapel Hill

For More Information
NCER Project Abstract and Reports:

http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/251/report/0

Peer Reviewed Publications

Styblo, M., Serves, S.V., Cullen, W.R., and Thomas, D.J. 1997. Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. Chemical Research in Toxicology 10(1):27-33.

Styblo, M., Del Razo, L.M., LeCluyse, E.L., Hamilton, G.A., Wang, C., Cullen, W.R., and Thomas, D.J. 1999. Metabolism of arsenic in primary cultures of human and rat hepatocytes. Chemical Research in Toxicology 12:560-565.

Styblo, M., Del Razo, L.M., Vega, L., Germolec, D.R., LeCluyse, E.L., Hamilton, G.A., Reed, W., Wang, C., Cullen, W.R., and Thomas, D.J. 2000. Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in human cells. Archives of Toxicology 74(6):289-299.

Del Razo, L.M., Styblo, M., Cullen, W.R., and Thomas, D.J. 2001. Determination of trivalent methylated arsenicals in biological matrices. Toxicology and Applied Pharmacology 174(3):282-293.

Lin, S., Del Razo, L.M., Styblo, M., Wang, C., Cullen, W.R., and Thomas, D.J. 2001. Arsenicals inhibit thioredoxin reductase in cultured rat hepatocytes. Chemical Research in Toxicology 14(3):305-311.

Styblo, M., and Thomas, D.J. 2001. Selenium modifies the metabolism and toxicity of arsenic in primary rat hepatocytes. Toxicology and Applied Pharmacology 172(1):52-61.

Thomas, D.J., Styblo, M., and Lin, S. 2001. Review: The cellular metabolism and systemic toxicity of arsenic. Toxicology and Applied Pharmacology 176(1):127-144.

Vega, L., Styblo, M., Patterson, R., Cullen, W., Wang, C., and Germolec, D. 2001. Differential effects of trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in normal human epidermal keratinocytes. Toxicology and Applied Pharmacology 172(3):225-232.

Lin, S., Shi, Q., Nix, F.B., Styblo, M., Beck, M.A., Herbin-Davis, K.M., Hall, L.L., Simeonsson, J.B., and Thomas, D.J. 2002. A novel S-adenosyl-L-methionine: Arsenic(III) methyltransferase from rat liver cytosol. Journal of Biological Chemistry 277(13): 10795-10803.

Styblo, M., Drobna, Z., Jaspers, I., Lin, S., and Thomas, D.J. 2002. The role of biomethylation in toxicity and carcinogenicity of arsenic: A research update. Environmental Health Perspectives 110(5):767-771.