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Exposure to monomethylarsonous acid (MMA^{III}) leads to altered selenoprotein synthesis in a primary human lung cell model

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Abstract

Monomethylarsonous acid (MMA^{III}), a trivalent metabolite of arsenic, is highly cytotoxic and recent cell culture studies suggest it might act as a carcinogen. The general consensus of studies indicates the cytotoxicity of MMA^{III} is a result of increased levels of reactive oxygen species (ROS). A longstanding relationship between arsenic and selenium metabolism has led to the use of selenium as a supplement in arsenic exposed populations, however the impact of organic arsenicals (methylated metabolites) on selenium metabolism is still poorly understood. In this study we determined the impact of exposure to MMA^{III} on the regulation of expression of TrxR1 and its activity using a primary lung fibroblast line, WI-38. The promoter region of the gene encoding the selenoprotein thioredoxin reductase 1 (TrxR1) contains an antioxidant responsive element (ARE) that has been shown to be activated in the presence of electrophilic compounds. Results from radiolabeled selenoproteins indicate exposure to low concentrations of MMA^{III} resulted in increased synthesis of TrxR1 in WI-38 cells, and lower incorporation of selenium into other selenoproteins. MMA^{III} treatment led to increased mRNA encoding TrxR1 in WI-38 cells, while lower levels of mRNA coding for cellular glutathione peroxidase (cGpx) were detected in exposed cells. Luciferase activity of TrxR1 promoter fusions increased with addition of MMA^{III}, as did expression of a rat quinone reductase (QR) promoter fusion construct. However, MMA^{III} induction of the TRX1 promoter fusion was abrogated when the ARE was mutated, suggesting that this regulation is mediated via the ARE. These results indicate that MMA^{III} alters the expression of selenoproteins based on a selective induction of TrxR1, and this response to exposure to organic arsenicals requires the ARE element.

Keywords

Monomethylarsonous acid; thioredoxin reductase; antioxidant response element; arsenic; selenium; carcinogenesis

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Conflict of Interest Statement

No conflicts of interest.

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Introduction

Arsenic exposure is a worldwide public health concern. Mammals metabolize arsenic primarily in the liver by a series of methylation reactions (Styblo *et al.*, 2000; Thomas *et al.*, 2004; Li *et al.*, 2005; Thomas *et al.*, 2007). One of these methylated metabolites, MMA^{III}, has been shown to contribute to carcinogenesis, and exposure to MMA^{III} has led to mitogenic signaling critical to transformation (Bredfeldt *et al.*, 2006; Eblin *et al.*, 2007; Eblin *et al.*, 2008). MMA^{III} has been shown to be the most cytotoxic of the trivalent arsenicals (Petrick *et al.*, 2000), and has also been shown to cause lipid peroxidation, protein carbonylation, and oxidative DNA damage in cell culture studies (Wang *et al.*, 2007a). MMA^{III} exposure to cells in culture induced hyperproliferation, anchorage-independent growth, and tumorigenicity in an immortalized human urothelial cell line (Bredfeldt *et al.*, 2006). The results from this study led to a suggestion by the authors that MMA^{III} be classified as a carcinogen.

Selenocysteine is the 21st amino acid and is encoded by a stop codon, UGA. Selenoprotein synthesis is a complex process involving a myriad of proteins required for insertion of selenium into the final selenoprotein product (see (Papp *et al.*, 2007) for a thorough review). One of the factors required for selenoprotein synthesis is the selenocysteine insertion sequence (SECIS), a stem-loop structure in the 3' untranslated region of the mRNA (Berry *et al.*, 1993). Another protein involved is SECIS binding protein 2 (SBP2). SBP2 is required for efficient selenoprotein synthesis, and may help to recruit the ribosome to the UGA codon (Copeland *et al.*, 2000). Under selenium deficient conditions, some selenoproteins are preferentially produced over others in what is known as the selenoprotein hierarchy (Behne *et al.*, 1988). At the top of this hierarchy is thioredoxin reductase 1 (TrxR1) and phospholipid hydroperoxide glutathione peroxidase (PHGPx). Other selenoproteins such as cellular glutathione peroxidase (cGPx) are produced in selenium replete conditions (Berry, 2005). The mechanism of this hierarchy is beginning to be understood at the molecular level. Because it uses a stop codon, selenoprotein-coding mRNAs are sensitive to nonsense-mediated decay (NMD) (de Jesus *et al.*, 2006). NMD is in part determined by the introns present prior to the UGA codon. Another important factor is the efficiency of binding of SBP2 to the SECIS element. SBP2 has been shown to prefer certain SECIS elements over others (Low *et al.*, 2000). Thus, SBP2 likely has a key role in the regulation of this hierarchy of expression of selenoproteins.

Thioredoxin reductase (TrxR) has three primary isoforms. Expression of cytosolic selenoprotein TrxR1 is regulated at the transcriptional level through an antioxidant response element (ARE) in its promoter (Hintze *et al.*, 2003b). The ARE target sequence allows transcription of TrxR1 to be controlled by the Nrf2/Keap1 system. Many electrophilic compounds can induce the Nrf2/Keap1 response. In particular, it was found that sulforaphane induced transcription of the gene encoding TrxR1 through this system (Hintze *et al.*, 2003b; Zhang *et al.*, 2003). TrxR1 is expressed at higher levels in many tumors derived from breast, thyroid, prostate, liver, malignant melanoma, and colorectal tissue, as compared to normal tissues (Berggren *et al.*, 1996; Gladyshev *et al.*, 1998). Cytosolic thioredoxin (Trx), the primary substrate of TrxR1, is required for DNA synthesis, and is thus integral to cell proliferation (Laurent *et al.*, 1964). Ras oncogene activation has recently been shown to trigger activation of elevated levels of TrxR1, leading to a proliferative phenotype (Yoo *et al.*, 2007). It has been suggested that TrxR1 not only be a biomarker for cancer, but a potential target for cancer therapy (Arner and Holmgren, 2006). Several recent studies have shown that trivalent arsenicals are potent inhibitors of TrxR (Holmgren, 1977; Lin *et al.*, 2001; Lu *et al.*, 2007), and one of the most widely used therapies for acute promyelocytic leukemia is arsenic trioxide (ATO). ATO has been shown to act both as a potent inhibitor of TrxR1 (Lu *et al.*, 2007) and to block selenium metabolism (Talbot *et al.*, 2008), although it is not yet known whether these properties are underlying the molecular mechanism of the drugs efficacy for cancer treatment.

Inorganic arsenic and selenium are known to interact in what is known as the mutual sparing effect, first described by Moxon during studies of seleniferous grains (Moxon, 1938). This mutual sparing effect has also been demonstrated in animal and cell culture studies in which levels of inorganic selenium and arsenic were varied (Levander, 1977; Styblo and Thomas, 2001). A key finding in the interaction of selenium and arsenic was the discovery of the seleno-bis(S-glutathionyl) arsinium ion (Gailer *et al.*, 2002a; Gailer *et al.*, 2002b). This was shown to be excreted in the bile of rabbits treated with selenite and arsenate or arsenite (Gailer *et al.*, 2002). Recently we analyzed the affect of trivalent arsenicals on selenoprotein synthesis using a keratinocyte cell culture model (Ganyc *et al.*, 2007). It was found that arsenite decreased selenium incorporation into selenoproteins. In contrast, MMA^{III} induced selenium incorporation into TrxR1, while decreasing selenium incorporation into smaller selenoproteins, such as cGpx. Currently, there is no knowledge of a selenium and MMA^{III} complex formed, thus it appears that exposure to MMA^{III} leads to changes in the expression pattern of the genes encoding selenoproteins. To further understand the mechanism in this study we investigate the mechanism by which MMA^{III} affects selenoprotein synthesis using a primary lung fibroblast model (WI-38). The data reveal that exposure to MMA^{III} leads to significant increases in the mRNA encoding TrxR1 at the expense of cGpx. This increase expression requires the ARE element, suggesting that the differential expression of selenoproteins in response to MMA^{III} is due to activation of the Nrf2 pathway.

Materials and Methods

Materials

Sodium selenite was obtained from Acros Organics (Geel, Belgium). Radioisotope ⁷⁵Se was purchased from University of Missouri Research Reactor (MURR, Columbia, MO). MMA^{III} was obtained from the laboratory of Dr. William Cullen (University of British Columbia, Vancouver, Canada).

Cell culture

To determine the response to exposure to MMA^{III} within a target tissue (lung) a normal lung fibroblast (WI-38) culture was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultivated in Eagle's Minimum Essential Medium (EMEM) with Earle's balanced salt solution, non-essential amino acids, sodium pyruvate, 10% FBS, 50 µg/mL streptomycin, and 50 IU/mL penicillin. Culture monolayers were maintained at 37°C, in a humidified atmosphere containing 5% CO₂. Population doublings of WI-38 cells were determined as previously described (Hayflick, 1985). No cultures beyond a population doubling of 34.0 were used in this study to avoid the potential impact of cellular senescence.

Cell viability assay

Cells were plated at a concentration of 5,000 per well in 96-well culture dishes, and treated in triplicate with varying concentrations of MMA^{III}; 0.2, 0.5, 1, 3, 6, 9, 12, 15, 18, 21, 24, 28 µM. After 24 hours of incubation, the tetrazolium dye, 3-(4,5 dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) (Amresco, Solon, Ohio) was added to a final concentration of 1.2 mM and cells were subsequently incubated for 4 hours at 37°C. The formazan product of the dye was solubilized by addition of SDS (10%) and HCl (5 mM) followed by overnight incubation at 37°C. Absorbance of the reduced MTT was measured at 570 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). Cell viability was determined by dividing the absorbance of treated samples to untreated controls and reported as a percentage with error being the standard deviation from triplicate wells.

Radioisotope labeling to follow selenoprotein synthesis

Cells were cultured in 6-well tissue culture plates and treated at a density of 70% confluence. WI-38 cells were treated with 0, 0.2, 1, or 2 μM MMA^{III} in triplicate wells for 24 hours. In all wells radioisotope selenium (2 μCi of ⁷⁵Se), in the form of selenite (10 nM), was added to follow the metabolism of selenium.

Cells were harvested using trypsin-EDTA, collected by centrifugation, and subsequently washed with Dulbecco's modified phosphate buffered saline (DPBS). Cell pellets were resuspended in 200 μL of lysis buffer (50 mM tricine, pH 8.0, 0.1 mM benzamidine, 0.5 mM EDTA, and 1 mM DTT) and lysed by sonication for 6–8 sec using a Model 100 sonic dismembrator (Thermo Fisher Scientific, Pittsburgh, PA) at a power setting of 4 watts. Crude cell lysates were obtained by centrifugation at 11,000 rpm for 7 minutes at 4°C. Radiolabeled selenium was analyzed in lysates using a Wallac Wizard Gamma Counter, Model 1470 (PerkinElmer, Wellesly, MA). Protein concentration was determined by Bradford assay (Bradford, 1976) with bovine serum albumin used to generate a standard curve. For visualization of labeled selenoproteins 25 μg of protein from crude cell extracts was separated by SDS-PAGE (15%). The gel was stained using Imperial Protein Stain (Pierce, Rockford, IL) and subsequently dried prior to exposure to a phosphor screen. Labeled proteins were analyzed using a Storm Molecular Dynamics phosphorimager (Sunnyvale, CA) and densitometry was carried out using ImageQuant software.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

WI-38 cells were cultured in 25 cm² flasks, and grown to approximately 70% confluence. Cells were treated with 0, 0.2, or 2 μM MMA^{III}. Cells were harvested as described above with the exception that DPBS was supplemented with diethylpyrocarbonate (DEPC, 0.1%). Cells were resuspended in DPBS containing DEPC and RNA was isolated using a ChargeSwitch total RNA cell kit (Invitrogen, Carlsbad, CA). RNA was quantified by UV-visible spectrophotometry at 260 nm using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA). Complementary DNA (cDNA) was synthesized from 0.5 μg of RNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA).

Oligonucleotides utilized for real-time RT-PCR were as follows (listed forward then reverse): TrxR1; 5'-AGCTCAGTCCACCAATAGTGA-3' and 5'-GGTATTTTCCAGTCTTTTCAT-3'; HO-1; 5'-GTCTTCGCCCCCTGTCTACTTC-3' and 5'-CTGGGCAATCTTTTGTAGCAC-3'; cGPx; 5'-GGACTACACCCAGATGAA-3' and 5'-CAAGGTGTTCCCTCCCTCGTAG-3'; β -actin; 5'-CATGTACGTTGCTATCCA-3' and 5'-CTCCTTAATGTCACGCACGAT-3'. Real time PCR reactions were carried out using a Bio-Rad I-Cycler (Bio-Rad). Each reaction was a total volume of 25 μL consisting of SYBRgreen supermix (Bio-Rad) with each oligonucleotide at a concentration of 0.2 mM, forward and reverse primers (at a concentration of 0.2 μM each), and 5 μL of 1:100 diluted cDNA. Reaction conditions were as follows; one cycle at 95.0°C for 3 minutes, followed by 40 cycles of 95.0°C for 10 sec, and 55.0°C for 45 sec. With the cGPx-specific primers the following reaction conditions were used; a single cycle at 94.0°C for 3 minutes, subsequent 40 cycles of 94.0°C for 30 sec, 55°C for 30 sec, and 70.0°C for 30 sec. Melt curves and agarose gel electrophoresis were used to verify formation of a single product. Efficiency of amplification of each primer pair was calculated using a 10-fold dilution series with untreated cDNA. The Pfaffl method (Pfaffl, 2001) was used to determine the relative expression (reported as fold increase or decrease) for each of the mRNAs of interest using β -actin as an internal standard.

TrxR Activity Assays

WI-38 cells were cultured in 75 cm² flasks to generate sufficient material for TrxR enzyme assays. Cells were treated with 0, 0.2, or 2 μM MMA^{III} for 48 hours before harvesting as

described above. Cells pellets were resuspended in 500 μ L of lysis buffer (5 mM potassium phosphate pH 7.4, 0.5 mM EDTA). Cells were lysed by sonication as described above followed by centrifugation (10,000 \times g) for 7 min at 4 $^{\circ}$ C to yield crude cell extracts. After protein determination, cells extracts were diluted in a buffer consisting of 10 mM potassium phosphate pH 7.4, 154 mM potassium chloride, and 1 mg/mL of bovine serum albumin (BSA) to a final concentration of 1 mg/mL. TrxR assays were carried out as previously described (Smith and Levander, 2002) using 50 μ g of cell extract to start the reaction. Auranofin was used to treat cell extracts (20 minutes) to determine the gold-inhibited activity that is subtracted from the total reductase activity. Reduction of DTNB was followed at a wavelength of 412 nm at 37 $^{\circ}$ C for 3 minutes taking a reading every 15 seconds using a SpectraMax 190 spectrophotometer (Molecular Devices). Activity of TrxR is reported as nmol/min/mg of protein as previously described (Smith and Levander, 2002).

Promoter fusion luciferase assays

Luciferase promoter fusion constructs of human wild-type TrxR1, mutant (Nrf2 binding site mutant) TrxR1, and rat quinone reductase (QR) have been previously described (Hintze *et al.*, 2003b). Cells were seeded in 24-well tissue culture plates at 60,000 per well in 500 μ L of EMEM. Transfection complexes were prepared with either 1 μ g (TrxR1 or TrxR1 mutant construct) or 2 μ g (for QR construct) of plasmid DNA. For formation of transfection complexes 7.5 μ L Superfect reagent (Qiagen, Valencia, CA) was added per μ g of plasmid DNA, 540 μ L Optipro serum free medium (Invitrogen), and 2.5 ng of a Renilla luciferase internal control plasmid pRL-SV40 (Promega, Madison, WI) were mixed, added to cells and incubated for 3 hours. The culture medium was then removed and MMA^{III} was added in the following concentrations in triplicate; 0, 0.2, or 2 μ M. Cells were then incubated for 24 hours before assaying for luciferase activity. Luciferase assay was carried out using the dual luciferase reporter kit (Promega, Madison, WI) according to the manufacturer's instructions with the exception that passive lysis buffer was used at 2X concentration and cells were manually scraped from the plate to enhance cell lysis. A Glomax luminometer (Promega) was used to measure luminescence. Luciferase activity is reported using Renilla luciferase as a baseline for transfection efficiency. The ratio of Firefly luciferase to Renilla luciferase in untreated cells is set to an arbitrary value of 1.0.

Statistical Analysis

All statistical analyses were performed using Microsoft Excel. Unpaired t-tests were performed to test for statistical significance.

Results

Cytotoxicity of MMA^{III} in WI-38 cells

WI-38 cells are a primary lung fibroblast, which behave accordingly to the Hayflick model, and have been used extensively for studies on cellular senescence (Hayflick and Moorhead, 1961; Place *et al.*, 2005). Our goal in this study is to determine the effects of MMA^{III} on selenoproteins in a primary cell line, using a sub-toxic concentration of MMA^{III}. This cell line was chosen since the rigorous assessment of the effects to selenium metabolism by MMA^{III} are best determined with the use of primary cells. To determine a sub-toxic dose of MMA^{III}, we determined the cytotoxicity of MMA^{III} in WI-38 cells by MTT assay. A 50% decrease in cell viability was observed in cells treated with 6 μ M MMA^{III} (Figure 1). Treatment of cells with concentrations at or below 2.5 μ M MMA^{III} did not significantly reduce viability. Indeed low levels of MMA^{III} stimulated the reduction of MTT by these cells, approximately 20% above untreated controls. This stimulatory effect has been reported previously (Ganyc *et al.*, 2007), yet the mechanism behind it remains elusive. Nonetheless for this study we evaluated

the effects of MMA^{III} on selenium metabolism at concentrations at or below 2 μ M in all subsequent experiments.

Effect of MMA^{III} exposure on selenoprotein synthesis

To determine the effects of MMA^{III} on selenium metabolism and/or selenoprotein synthesis, cells were exposed to MMA^{III} and simultaneously labeled with ⁷⁵Se (selenite) radioisotope. Increasing exposure to MMA^{III} led to an increase in a prevalent band of molecular weight near 60 KDa (Figure 2). Immunoblot analysis of cell extracts confirmed that this is TrxR1 (data not shown). Incorporation of selenium into other selenoproteins decreased as incorporation into TrxR1 increased. Further analysis of the densitometry of the TrxR1 band revealed a two-fold increase with exposure to 1 and 2 μ M MMA^{III} compared to control (data not shown). The densitometry analysis of one of the smaller selenoproteins (denoted with an asterisk) showed a more than two-fold decrease with increasing exposure to MMA^{III}. This pattern is similar to that seen when HaCat cells were exposed to MMA^{III} (Ganyc *et al.*, 2007). Incorporation of selenium into newly synthesized TrxR1 increased at the expense of other selenoproteins. To further analyze the mechanism for this phenotype, we determined whether this regulation occurred at the level of transcription.

MMA^{III} increases TrxR1 mRNA levels in WI-38 cells

We next we determined whether any changes occur to the mRNA encoding TrxR1 or cGPx (a selenoprotein known to have a lower hierarchy status) by using real time RT-PCR. A significant increase in mRNA levels of TrxR1 (*, $p < 0.05$) (Figure 3A) was observed in WI-38 cells treated with 2 μ M MMA^{III}. However, treatment of cells with 0.2 μ M MMA^{III} did not elicit the same effect. This correlates well to the radiolabeling data in which exposure to 2 μ M MMA^{III} resulted in increased levels of TrxR1.

Likewise, treatment of WI-38 cells with MMA^{III} also caused mRNA levels of cGPx to decrease (Figure 3B). In cells exposed to 2 μ M MMA^{III}, there was a significant decrease in cGPx mRNA (*, $p < 0.05$). This also correlates with the selenium radiolabeling data in this cell line. Specifically, there was a decrease in selenium incorporation into selenoproteins other than TrxR1 with exposure to MMA^{III} (Figure 2).

Exposure to MMA^{III} reduces TrxR activity in WI-38 cells

It is well established that MMA^{III} and other trivalent arsenicals are potent inhibitors of TrxR when assayed using purified enzyme (Lin *et al.*, 1999). In addition exposure to trivalent arsenicals led to decreases in TrxR activity in cultured hepatocytes (Lin *et al.*, 2001). To determine whether MMA^{III} exposure is affecting TrxR activity, cells were cultured with MMA^{III} for 48 hours. Cells were subsequently harvested and TrxR activity was determined by DTNB assay (Smith and Levander, 2002; Hintze *et al.*, 2003a). Treatment with 0.2 μ M MMA^{III} led to an increase in the activity of TrxR above that of control (from 9.88 ± 0.290 to 14.7 ± 1.19 nmol/min/mg). In contrast, when cells were cultured in the presence of 2 μ M MMA^{III}, TrxR activity decreased as compared to control (from 9.88 ± 0.290 to 6.33 ± 0.212 nmol/min/mg). Although there is increased incorporation of selenium into TrxR1, the enzyme's activity has been reduced, likely mediated by direct inhibition with the arsenical as observed in a previous study (Lin *et al.*, 1999).

MMA^{III} exposure results in activation of transcription of the TrxR1 promoter

The TrxR1 promoter contains an ARE along with binding sites for Oct-1, Sp1, and Sp3 (Rundlof *et al.*, 2001; Hintze *et al.*, 2003a). To test if MMA^{III} induces synthesis of TrxR1 through the ARE, promoter fusion luciferase constructs were transfected into WI-38 cells. This included a wild type TrxR1 promoter containing the ARE as well as a second construct with

a mutated (Mut) ARE element. If exposure to MMA^{III} exerts any effects on the activation of the ARE of TrxR1 by Nrf2, the same effects should not be seen in the mutated construct.

WI-38 cells transfected with the wild-type promoter fusion construct displayed increased luciferase activity upon exposure to 2 μM MMA^{III} (*, $p < 0.05$) (Figure 4). There was a slight increase in activity in cells treated with 0.2 μM MMA^{III}, but not enough to be considered statistically significant. No significant change in luciferase activity was observed in WI-38 cells transfected with the mutant promoter fusion construct and exposed to 0.2 and 2 μM MMA^{III}, clearly indicating that exposure to MMA^{III} is leading to activation of the TrxR1 promoter via the ARE. Based on other studies, this activation is likely mediated through the Nrf2 transcriptional activator (Aono *et al.*, 2003; Pi *et al.*, 2003; Wang *et al.*, 2007b).

Activation of transcription of the promoter for quinone reductase in WI-38 cells by MMA^{III}

The gene encoding quinone reductase also contains an ARE in its promoter region, and this gene product is not known to be involved in selenium metabolism (Hintze *et al.*, 2003b). WI-38 cells were transfected with a rat quinone reductase (QR) promoter fusion construct that contained the ARE. In WI-38 cells transfected with the QR construct and exposed to varying concentrations of MMA^{III}, an increase in QR promoter fusion activity was detected upon exposure to 2 μM MMA^{III} (Figure 5). There was also a slight increase in luciferase activity with treatment of cells with 0.2 μM MMA^{III}, again not enough to be considered significant.

In addition to the induction of QR, we also examined the level of mRNA encoding heme oxygenase 1 (HO-1), another gene whose promoter contains an ARE, during exposure to MMA^{III}. At treatment of cells with 2 μM MMA^{III} there was ten-fold induction of mRNA levels encoding HO-1 as determined by RT-PCR (data not shown). This provides further evidence that treatment of cells with MMA^{III} respond through the ARE and this response is very likely to be mediated by the Nrf2 response.

Discussion

MMA^{III} is a metabolite of arsenic produced during the methylation reactions carried out to facilitate excretion. It is not found in the natural environment, but is generated during this excretory pathway. It has been found to be the most cytotoxic of all arsenicals and could prove to be carcinogenic (Petrick *et al.*, 2000; Bredfeldt *et al.*, 2006). Human exposure to arsenic is primarily through inorganic forms, especially arsenate (Nickson *et al.*, 1998). A recent study has shown that exposure to mice to arsenate (50 ppm in water) for 12 weeks led to an accumulation of MMA^{III} in lung tissue of 0.1 $\mu\text{g}/\text{gm}$ of tissue (Kenyon *et al.*, 2008). This level (0.1 ppm) is comparable to exposure to cells at 1 μM . Our experiment design, since it follows incorporation of radioisotope selenium, is limited to short term experiments. Nonetheless we see a significant shift in the synthesis of selenoprotein synthesis at exposure levels (2 μM) very close to those seen in animal studies (Kenyon *et al.*, 2008). Since humans are usually exposed for far longer than 12 weeks, our results and experimental design are clearly relevant to human exposure. Furthermore many studies have utilized exposures to methylated arsenicals in the micromolar range to elicit cellular responses in the short term (Styblo *et al.*, 2000; Lin *et al.*, 2001; Liu *et al.*, 2001). These studies also have shown that significant methylation of arsenicals is exhibited by hepatocytes and cells of the renal system, with little methylation of arsenic occurring in other cell types including fibroblasts (Wang and Lazarides, 1984; Styblo *et al.*, 2000).

It has been shown that selenium and arsenic interact according to a mutual sparing effect (Moxon, 1938). Based on several studies it is well established that exposure to mammals to inorganic arsenic leads to decreased selenium levels (Levander and Argrett, 1969; Levander, 1977). If selenium levels were depleted, the cell will begin to preferentially make some

selenoproteins over others (Low *et al.*, 2000). TrxR1, because of its importance in maintaining the cellular redox environment is one of the selenoproteins at the top of the hierarchy and will likely be expressed under selenium limiting conditions. A recent study has demonstrated that administration of selenium to hairless mice significantly reduced the arsenic-enhanced carcinogenesis by UV radiation (Burns *et al.*, 2008). Thus it is clear that a thorough understanding of the effects of arsenic exposure on selenium metabolism is justified. Moreover culture model systems are a more direct way to establish how cells react to any of the metabolic intermediates in a controlled manner. In this study we aimed to determine whether exposure to MMA^{III} revealed any changes in selenium metabolism and selenoprotein synthesis in a primary cell, as well as determine the mechanisms involved.

When WI-38 cells were exposed to MMA^{III} TrxR1 synthesis increased, while smaller selenoproteins decreased as determined by radiolabeling with ⁷⁵Se. This phenotype with MMA^{III} exposure was also observed in a previous study with HaCat cells (human keratinocytes) (Ganyc *et al.*, 2007) yet in this work we determined the mechanism by which the incorporation into this particular selenoprotein occurs. This phenotype (i.e. increased TrxR1 levels with lower levels of cGpx) was also observed in an animal study studying the differential regulation of selenoproteins in response to sulforaphane (Hintze *et al.*, 2003a). This correlation suggested to us that the Nrf2 response was responsible for the differential regulation of expression of selenoproteins. In this study we demonstrate that MMA^{III} induces TrxR1 through the ARE, although it is not surprising since other trivalent arsenicals have been shown to induce an active Nrf2 response (Aono *et al.*, 2003; He *et al.*, 2006; Wang *et al.*, 2007b). It should be noted that induction of heme oxygenase, known to be induced by Nrf2, has been used as biomarker for arsenic exposure (Falkner *et al.*, 1993; Kenyon *et al.*, 2005). In one study with inorganic arsenic exposures in mice of up to 100 μmol/kg, significant increases in heme oxygenase was detected in liver and kidney but not in lung (Kenyon *et al.*, 2005). However a study using one dose of inorganic arsenic at 75 μmol/kg in guinea pigs did find an induction of heme oxygenase activity in lungs (Falkner *et al.*, 1993)..

Based on the results presented in this and several previous studies one can begin to determine the combined effects of arsenic exposure at the molecular level with respect to selenium metabolism and selenoenzyme activity. Specifically, 1.) exposure to arsenite will lead to reduction of selenium levels in tissues, due to the Moxon effect (Gailer *et al.*, 2002; Ganyc *et al.*, 2007; Burns *et al.*, 2008), 2.) Exposure to arsenite or MMA^{III} will lead to induction of the Nrf2 response (Aono *et al.*, 2003; Pi *et al.*, 2003; Wang *et al.*, 2007b), 3.) Synthesis of TrxR1 mRNA will increase in tissues exposed to either arsenite or MMA^{III}, mediated through the ARE, 4.) Higher expression of TrxR1 mRNA will lead to preferential production of TrxR1 over cGpx and other selenoproteins due to non-sense mediated decay of selenoproteins at a lower level on the hierarchy (de Jesus *et al.*, 2006; Small-Howard *et al.*, 2006), and 5.) TrxR activity will be inhibited by direct binding of trivalent arsenicals to the active site CXU motif of TrxR1 (Lin *et al.*, 1999; Lin *et al.*, 2001). Figure 6 is overview describing these cumulative effects. This combination will reduce significantly the cell's ability to defend against oxidative stress through decreases in Gpx isoenzymes and catalytic inactivation of the thioredoxin system. The thioredoxin system is needed not only for proliferation, but also as an electron donor for peroxiredoxins and methionine sulfoxide reductases (Moskovitz, 2005; Rhee *et al.*, 2005). Future studies to examine whether this series of events are the basis for the carcinogenic potential of arsenic in animal models is thus warranted.

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Abbreviations

MMA^{III}	Monomethylarsonous acid
TrxR	thioredoxin reductase
TrxR1	cytosolic thioredoxin reductase
QR	quinone reductase
ARE	antioxidant response element
cGpx	cytosolic glutathione peroxidase
PHGpx	phosphohydrolipid glutathione peroxidase

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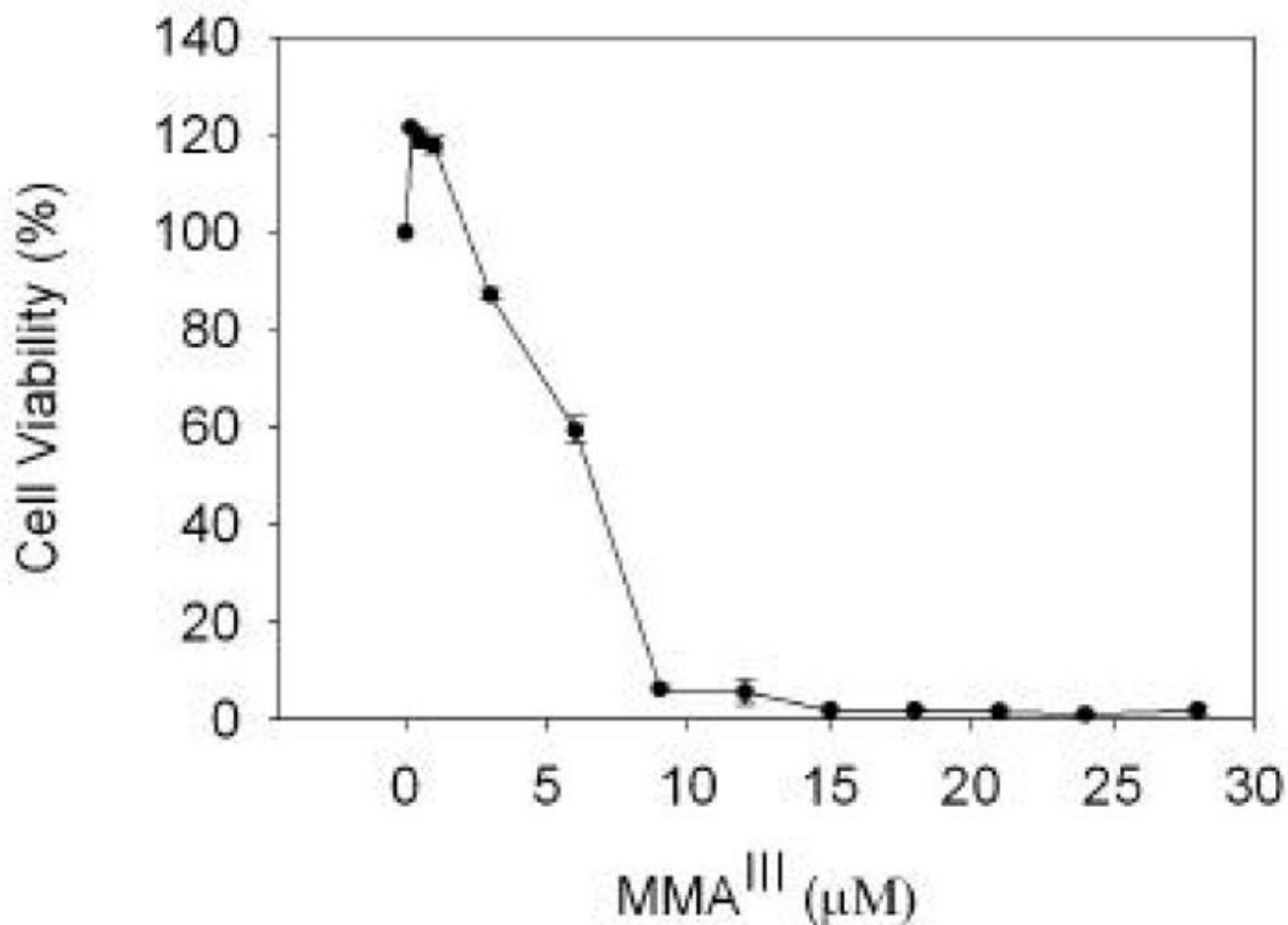


Figure 1. Cytotoxicity of MMA^{III} in WI-38 cells

Cells were treated with MMA^{III} for 24 hours and cell viability is reported as a percentage of viable cells based on untreated controls. The mean value is reported and error shown is the standard deviation from at least three independent cultures.

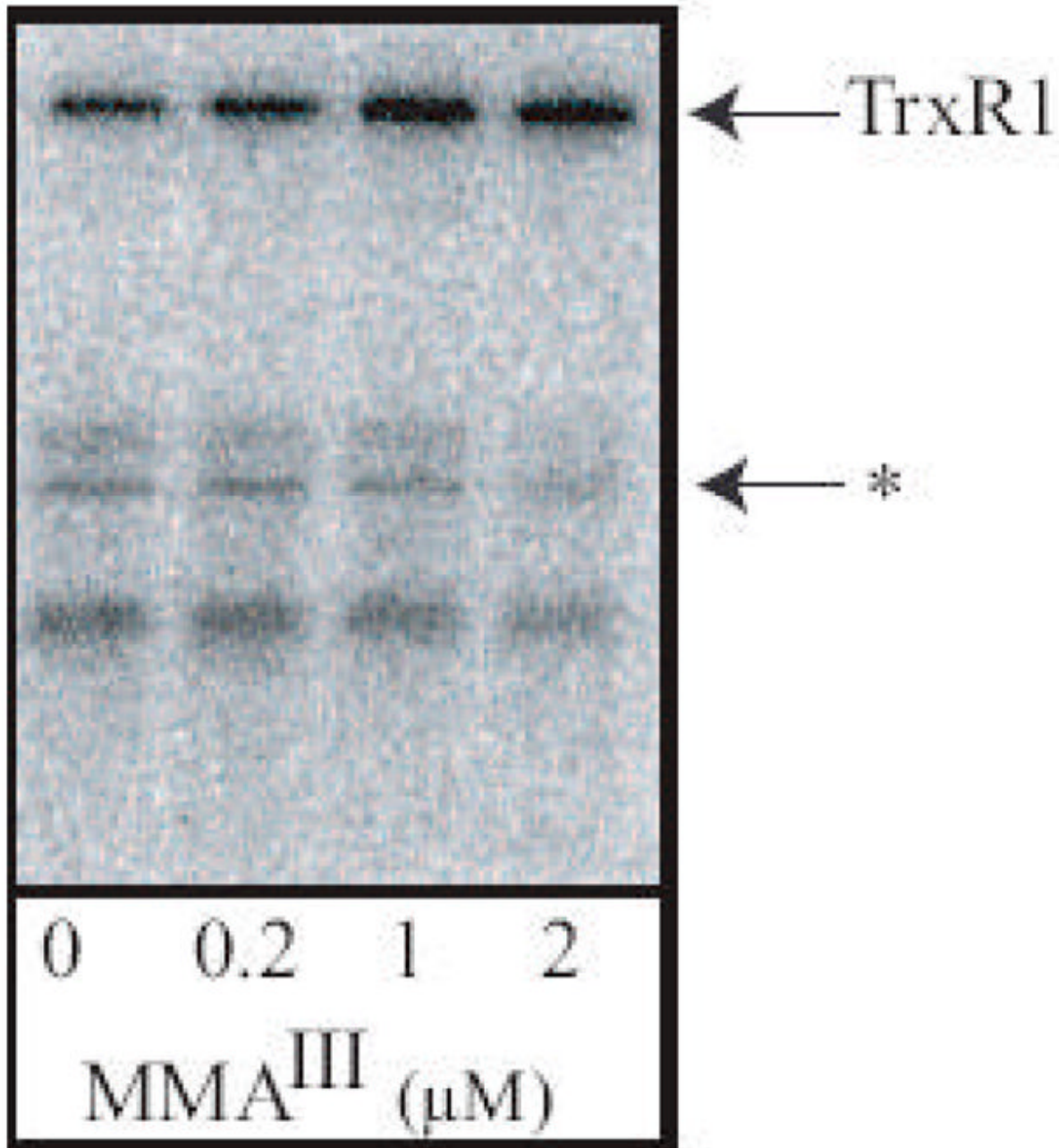


Figure 2. MMA^{III} treatment of WI-38 cells leads to increased synthesis of TrxR1 at the expense of other selenoproteins

WI-38 cells were treated with MMA^{III} at the concentrations indicated in triplicate. During exposure to MMA^{III}, ⁷⁵Se (selenite) was added to the culture medium and cells were incubated for 24 hours. Crude cell extracts (25 μg protein) were resolved by SDS-PAGE (15%) and labeled selenoproteins were visualized using phosphor screen. TrxR1 – cytosolic thioredoxin reductase; Arrow labeled with an asterisk indicates a selenoprotein used for analysis of selenium incorporation into proteins other than TrxR1.

Figure 3A.

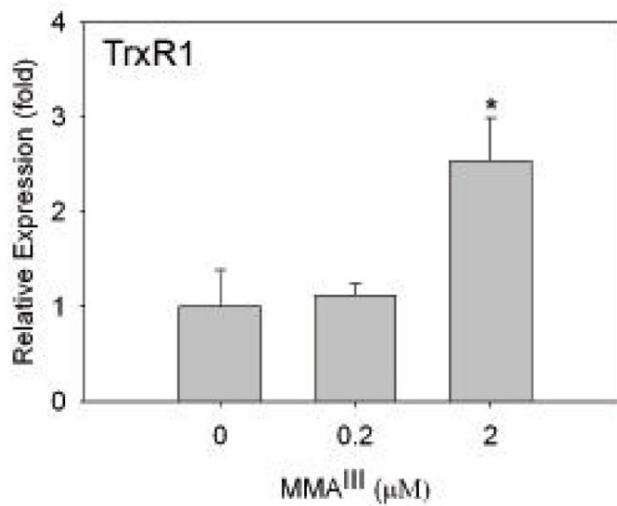


Figure 3B.

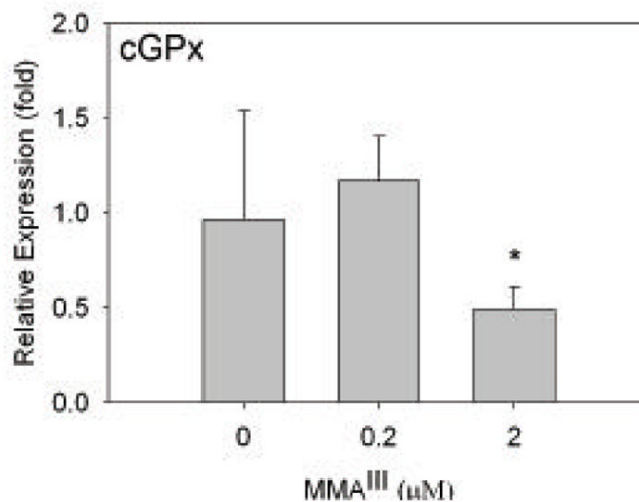


Figure 3. Exposure of WI-38 cells to MMA^{III} results in increases in TrxR1 mRNA levels
Cells were cultured with MMA^{III} for 24 hours after which cells were harvested for real-time RT-PCR analysis. β -actin was used as an internal standard. Relative expression plotted is a representative experiment (cultures in triplicate) with standard deviation as error. Statistical significance was determined by t-test. There is a significant difference in TrxR1 mRNA levels (*, $p < 0.05$) in WI-38 cells when treated with 2 μ M MMA^{III} (A). As TrxR1 mRNA levels are increasing with exposure to MMA^{III}, cGPx levels significantly decrease with treatment of 2 μ M MMA^{III} (*, $p < 0.05$) (B).

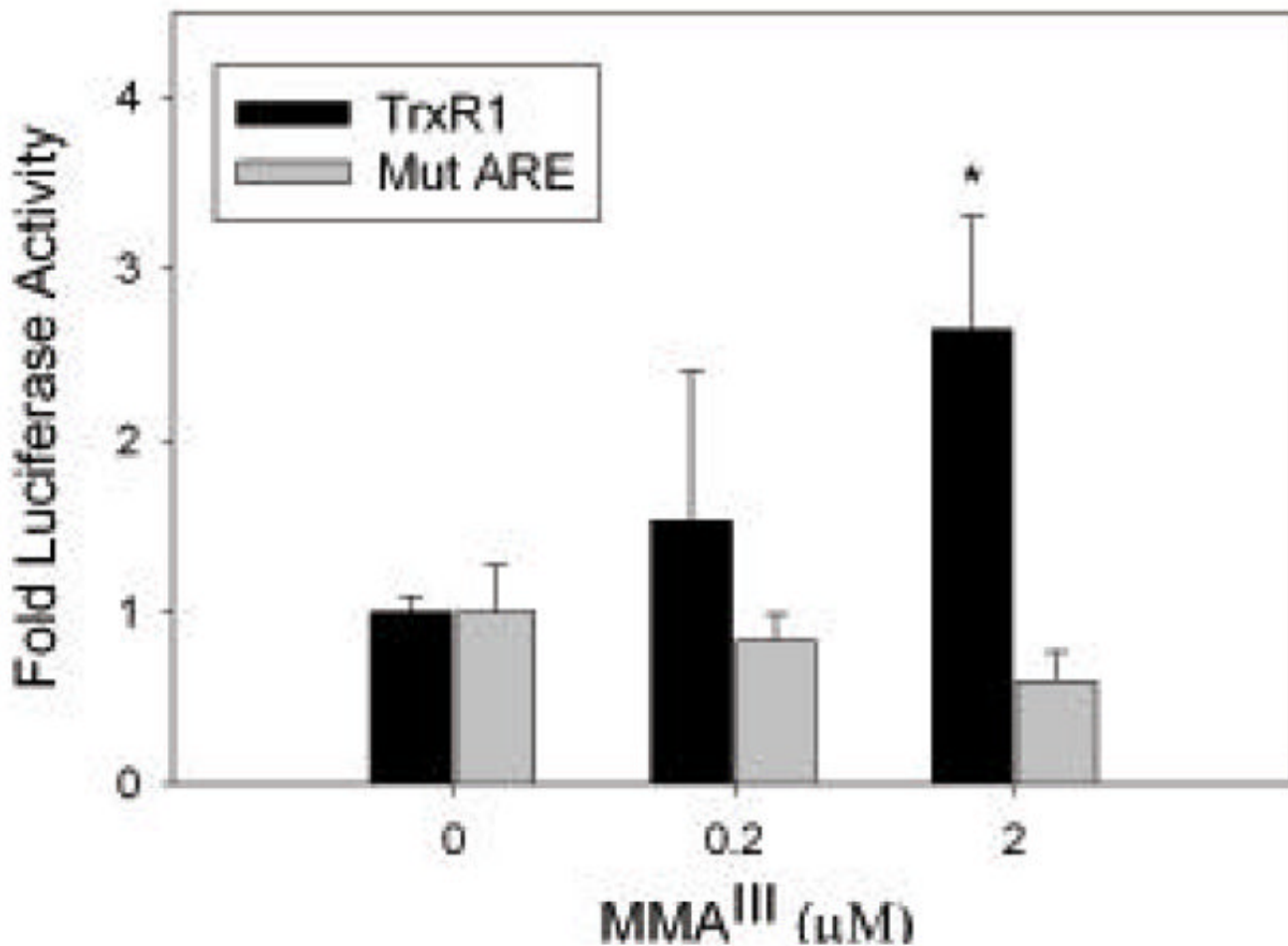


Figure 4. MMA^{III} treatment activates transcription of TrxR1 promoter through the ARE element WI-38 cells were transiently transfected with TrxR1 and mutant TrxR1 (mutation in ARE) promoter fusion constructs that have been previously described (Hintze *et al.*, 2003b). Cells were treated with MMA^{III} at the concentrations indicated in triplicate for each construct for 24 hours prior to assaying Firefly and Renilla luciferase activity. Fold luciferase activity is plotted and based on the ratio of Firefly luciferase to Renilla luciferase activity. The error bars represent standard deviation and student's t-test was used for statistical analysis. In WI-38 cells transfected with the TrxR1 construct and exposed to 2 μ M MMA^{III} there is a significant increase (*, $p < 0.05$) in luciferase activity. This increase in luciferase activity is not observed with the mutant ARE construct.

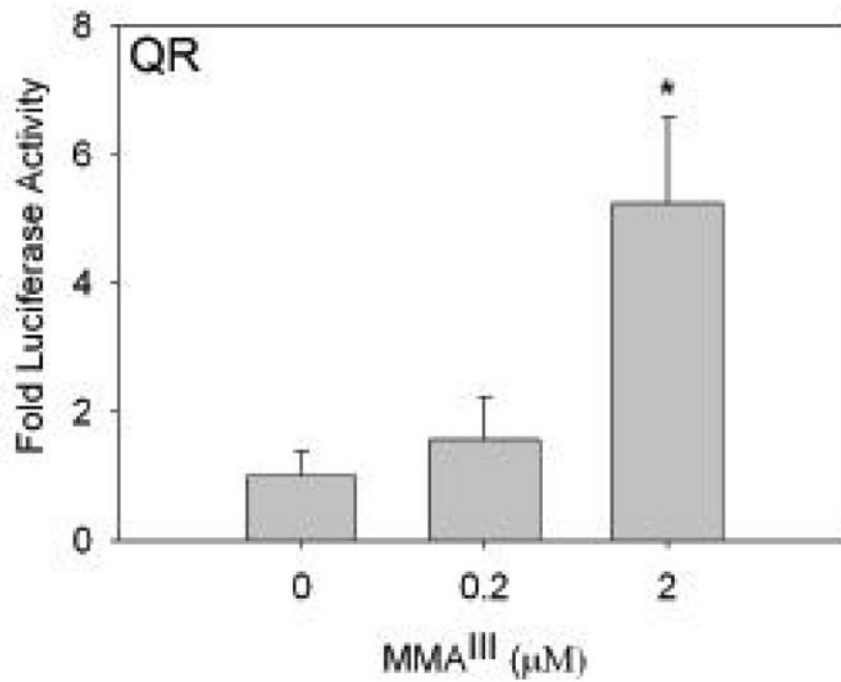


Figure 5. Treatment of WI-38 cells with MMA^{III} activates quinone reductase promoter confirming ARE regulation

WI-38 cells were transiently transfected with rat quinone reductase (QR) promoter fusion construct (Hintze *et al.*, 2003b) and exposed to MMA^{III} at the concentrations indicated. Similar to the results for TrxR1 construct, cells treated with 2 μM MMA^{III} increased activation of the promoter of QR (*, p<0.05).

Impacts of exposure for AsO_3^{2-} / MMA^{III} on Selenoenzymes

Exposure to arsenite:

↓ Selenium bioavailability (Moxon effect)

Exposure to MMA^{II} :

Activation of Nrf 2 response through ARE, leading to:

↓ Glutathione peroxidases (higher H_2O_2 and lipid peroxides)

↑ Incorporation of selenium into TrxR1 – preferential (hierarchy)

Direct inhibition of TrxR1 by either MMA^{II} or reduced metabolites of arsenite

Loss of TrxR1 activity leading to:

↓ Level of reduced thioredoxin leading to altered cellular redox potential

↓ Reduced activity of peroxiredoxins, leading to higher lipid and organic peroxides, peroxynitrite

Figure 6. Overview of the impact of arsenite and MMA^{III} on selenoenzymes

Arrows indicate positive or negative impact on several aspects of selenoenzymes and redox metabolism. Overview is based on data presented in this study and cumulative data from others (Lin *et al.*, 1999; Gailer *et al.*, 2000; Lin *et al.*, 2001; Rundlof *et al.*, 2001; Gailer *et al.*, 2002; Moskovitz, 2005; Rhee *et al.*, 2005; Ganyc *et al.*, 2007; Talbot *et al.*, 2008).