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Organophosphate pesticides increase the expression of alpha glutathione *S*-transferase in HepG2 cells

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ABSTRACT

Chlorpyrifos and methyl parathion are among the most widely used insecticides in the world. Human populations are constantly exposed to low doses of both due to their extensive use and presence in food and drinking water. Glutathione *S*-transferase (GST) catalyzes the conjugation of glutathione on electrophilic substrates and is an important line of defense in the protection of cellular components from reactive species. GST alpha1 (GSTA1) is the predominant isoform of GST expressed in the human liver; thus, determining the effect of insecticides on GSTA1 transcription is very important. In the present study, we analyzed the effects of methyl parathion and chlorpyrifos on GSTA1 gene expression in HepG2 cells using real time PCR, and activity and immunoreactive protein assays. The results demonstrated that exposure to methyl parathion and chlorpyrifos increased the level of GSTA1 mRNA, GSTA1 immunoreactive protein and GST activity relative to a control. These results demonstrated that these insecticides can increase the expression of GSTA1. In conclusion, HepG2 cell cultures treated with methyl parathion and chlorpyrifos could be a useful model for studying the function of GSTA1 and its role in the metabolism of xenobiotics in the liver.

1. Introduction

Organophosphate pesticides (OPs) such as chlorpyrifos (CP) and methyl parathion (MeP) are the most widely used insecticides in the world (Hreljac et al., 2008). OPs are typically esters, amides or thiol derivatives of phosphoric, phosphonic or phosphinic acids (Balali-Mood and Balali-Mood, 2008). Due to their widespread use, a large number of poisonings occur every year in occupationally-exposed human populations, and in the general population through the consumption of contaminated food and drinking water (Marrs, 1993; Yasmashita et al., 1997). CP and MeP are activated in the human liver by cytochrome P450-mediated oxidative desulfuration to form toxic oxygen analogs (oxons, P=O) (Sultatos et al., 1985; Sultatos, 1994). The oxon is the mediator of acute OPs toxicity due to its ability to inhibit acetylcholinesterase activity in the nervous system and neuromuscular junctions. Detoxification of the oxon occurs via hydrolysis by A-esterases or by conjugation with glutathione (GSH), which is catalyzed by glutathione *S*-transferase (GST) (Mutch et al., 2007; Jokanovic, 2001).

GST genes code for important phase II detoxification enzymes, which are an integral part of the defense mechanism that protects against cellular damage from chemical agents. GST metabolizes organic compounds such as carcinogens in tobacco smoke, and has both antioxidant and anti-inflammatory properties (Hayes et al., 2005). GSTs involved in OPs detoxification play an important role in developing resistance to insecticides (Abel et al., 2004; Fujioka and Casida, 2007). Mammalian GSTs have been grouped into eight isoforms, including alpha (α), kappa (κ), mu (μ), omega (ω), pi (π), sigma (σ), theta (θ) and zeta (ζ) (Hayes et al., 2005).

Transcriptional activation of GST by various xenobiotics remains an active area of research due to their important role in detoxification reactions (Rushmore and Pickett, 1990; Maglich et al., 2002). Recent studies revealed that OPs are able to regulate and induce CYP450 isoforms 3A4 and 2B6 through the pregnenolone X receptor (PXR) (Lemaire et al., 2004). However, the induction and regulation of GST genes by organophosphates such as CP and MeP have not yet been investigated. Because GSTA1 is the predominant isoform of GST expressed in the human liver, the evaluation of the effects of OPs on GSTA1 transcription is of relevance. The aim of the present study was to analyze the effects of CP and MeP on GSTA levels in HepG2 cells, a human hepatocarcinoma cell line.

2. Materials and methods

2.1. Materials

HepG2 cells were obtained from ATCC (Manassas, VA, USA), and CP and MeP were obtained from ChemService (West Chester, PA,

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USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl (MTT) and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). PCR reagents, probes and TaqMan Universal PCR Master Mix were purchased from Applied Biosystems Inc. (Foster City, CA, USA).

2.2. Cell culture and treatment

HepG2 cells were grown in Dulbecco's modified Eagle medium (Invitrogen Life Technologies, Carlsbad, CA, USA), which was supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), penicillin (100 U/ml), streptomycin (100 μg/ml), 1% L-glutamine and 1% nonessential amino acids (Invitrogen Life Technologies Carlsbad, CA, USA). Cell cultures were maintained in 75 cm^2 flasks at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. Cells were treated with different MeP and CP concentrations for 24, 48, 72 and 96 h. CP and MeP controls consisted of an equivalent volume of DMSO (0.05% v/v).

2.3. Metabolic activity

The cytotoxic effect of CP and MeP on HepG2 cells was evaluated using the MTT assay to measure metabolic activity (Mosmann, 1983). Briefly, 20 μl MTT (5 mg/ml in PBS) was added to each well, and the cells were incubated for 4 h. Then, 100 μl of 10% SDS in 0.01 N HCl was added to dissolve the MTT residue, and the optical density was determined at 570 nm on an ELISA-plate reader (μQuant Bio Tek, Vermont, USA).

2.4. Isolation of total RNA

Using Trizol, RNA was prepared from cultured HepG2 cells according to the manufacturer's instructions (Invitrogen). RNA was quantified spectrophotometrically at OD_{260}, and the purity was assessed by measuring the OD_{260}/OD_{280} ratio. The integrity of the RNA was evaluated by separating the RNA samples on 1% agarose gels. cDNA was prepared from 4 μg RNA, and the SuperScript Pre-amplification System for First Strand Synthesis (Invitrogen) and oligo dTs were used according to the manufacturer's instructions.

2.5. Real Time PCR detection

Quantitative real-time PCR assays (rtPCR) of the transcripts were performed with gene-specific fluorescent labeled probes on a 7000 Sequence Detector (Applied Biosystems). The probes were labeled with 6-carboxyfluorescein (FAM) and VIC as the 5′-fluorescent reporter. The following non-fluorescent quenchers at the 3′-end of the probes were designed using Primer Express software (Applied Biosystems): forward (5′- GGACGGTGACAGCGTTTAAC-3′), reverse (5'-GGCTTCTCTGCCATGATAGCA-3') and probe FAM (5′-AAAGCTTAGAGAAACCTCC-3′). The specificity of GSTA1 primers and the probe were verified by a lack of amplification from genomic DNA. Endogenous 18S, which is validated for stable expression in HepG2 cells, was used to normalize the mRNA data. The PCR reaction mixture contained 4 μl cDNA, 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and 0.9 μM and 0.25 μM of the primers and probe, respectively. PCR reactions were performed on an ABI PRISM 7000 sequence detector system (Applied Biosystems), and the following cycling protocol was employed: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min and 40 cycles at 95 °C and 60 °C for 15 s and 1 min, respectively. Each sample was analyzed twice in three independent experiments. The results were evaluated according to the comparative Ct method (Livak and Schmittgen, 2001).

2.6. Total protein determination

The total protein concentration of the cell lysates was measured with the Modified Lowry Protein Assay kit from Pierce (Pierce, Rockford IL, USA) according to the manufacturer's protocol. The total protein concentration was determined spectrophotometrically at 750 nm.

2.7. Western blot

HepG2 cells were harvested by scraping and were sonicated in 1.5 mL of buffer (20% glycerol, 100 mM Tris–HCl, 1 mM DTT and 200 mM PMSF) for 1 min. The resulting solution was centrifuged at 13,000 rpm for 3 h at 4 °C. The supernatant was isolated, and the concentration of protein was determined. The supernatant was diluted with buffer (2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.625 mM Tris–HCl (pH 6.8) and 0.001% bromophenol blue) in a boiling water bath for 5 min prior to electrophoresis. Aliquots containing 13 μg protein were applied to 12.5% SDS–polyacrylamide gels. The protein was transferred to a nitrocellulose membrane using a mini trans-blot system (Bio-Rad, Hercules, CA, USA). The transfer was performed at a constant voltage of 80 V for 2.5 h in a buffer consisting of 48 mM Tris–HCl, 39 mM glycine (pH 8.3) and 20% methanol at 4 °C. The membranes were blocked overnight at 4 °C in the presence of 5% nonfat dry milk and 0.5% bovine serum albumin (BSA) in blocking buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl) and were subsequently incubated at 4 °C for 7 h in a 1:5000 solution of human GSTA1-1 polyclonal antibody (Oxford Biomedicals, Rochester, MI) in sample buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 0.05% nonfat dry milk and 0.05% BSA). After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 3 h at room temperature. The membrane was washed, and the immunoreactive protein was detected using an ECL western blotting detection kit (Amersham, Arlington Heights, IL, USA). The integrated optical density of the bands was quantified using scanning densitometry.

2.8. GST enzymatic activity assay

Cells cultured as described above were treated with CP and MeP. The total GST activity was determined using a GST Assay Kit (Cayman Chemicals Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. The conjugation of 1-chloro 2,4-dinitrobenzene (CDNB) with reduced glutathione was measured spectrophotometrically (340 nm). Each sample was analyzed twice in three independent experiments.

2.9. Statistical analysis

The results are presented as the mean ± standard deviation (S.D.). Each experiment was performed in duplicate, and three independent experiments were conducted. The statistical significance, defined as a P value less than 0.05, between treated samples and the controls was determined by the Mann–Whitney test.

3. Results

3.1. Effect of CP and MeP on HepG2 metabolic activity

The cellular toxicity of CP and MeP was determined by the MTT assay. HepG2 cells were treated with CP and MeP at different concentrations (2–20 μM) for 24, 48, 72 or 96 h, and the cellular metabolic activity was evaluated. Cell cultures displayed a marked decrease in metabolic activity when treated with 6–20 μM CP and MeP for 72 or 96 h (Figs. 1A and 2B). These results are consistent

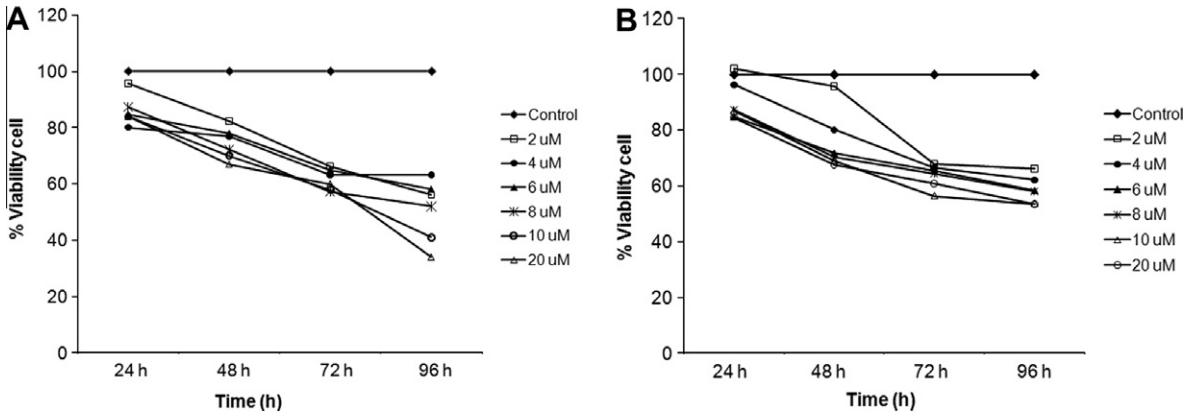

Fig. 1. The effect of chlorpyrifos (CP) and methyl parathion (MeP) on metabolic cellular activity. HepG2 cultures were treated with various concentrations of CP (A) or MeP (B) for the indicated time. At the end of each incubation period, cell viability was determined according to the MTT method. Data are given as the mean from three independent experiments (*$P < 0.05$ vs. control).

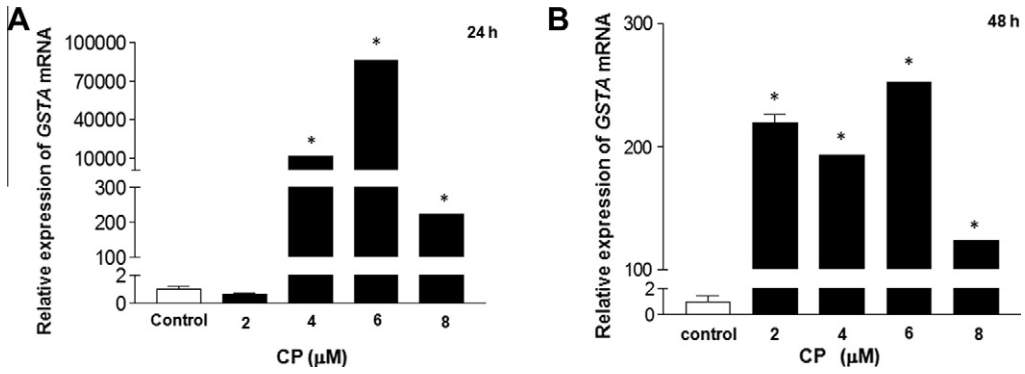

Fig. 2. Effects of chlorpyrifos (CP) on GSTA1 mRNA levels in HepG2 cells. HepG2 cells were treated with CP (2–8 μM) or 0.05% of DMSO (control). After 24 h (A) or 48 h (B) treatment, the relative expression of GSTA mRNA was determined by rtPCR. Changes in the level of expression were determined by normalizing the data to 18S and were expressed relative to the control. Data are given as the mean ± S.D. from two experiments performed in triplicate (*$P < 0.05$ vs. control).

with previous reports, which demonstrated that low concentrations of OPs do not affect cell viability. However, when cells were exposed to pesticide concentrations greater than or equal to 12 μM, cell viability decreased significantly in a concentration-dependent manner (Moore et al., 2010).

3.2. CP and MeP treatment increase GSTA1 mRNA expression in HepG2 cells

Compared to the controls, treatment with CP and MeP increased GSTA1 mRNA expression (Figs. 2A and B, 3A and B), with maximum induction achieved when the cells were treated with 6 μM CP. In particular, an 86,000-fold increase in GSTA1 mRNA levels was attained after 24 h (Fig. 2A). Similar but less pronounced effects were observed after 48 h (Fig. 2B). Alternatively, MeP exposure for 24 h did not increase GSTA1 mRNA levels (Fig. 3A). However, compared to the control, exposure to 8 μM MeP resulted in a 250-fold increase in GSTA1 mRNA levels at 48 h (Fig. 3B). These results indicate that both CP and MeP modulate mRNA levels of GSTA1 in HepG2 cells.

3.3. Induction of protein GSTA1 in HepG2 cells

We investigated whether the observed increase in GSTA1 mRNA levels reflected a change in GSTA1 protein expression. Western blot analysis indicated that treatment with 2 and 4 μM CP increased GSTA1 protein levels by 1.3 and 1.5-fold, respectively, after 24 h (Fig. 4A). Similar but less pronounced effects were observed with the 48 h treatments (Fig. 4B). However, treatment with MeP did not increase protein levels (Fig. 5A and B). This result may be due to the fact that the observed increase in GSTA mRNA levels was detected after 48 h, and an increase in protein expression may occur at 72 h. However, this hypothesis could not be verified due to the lower percentage of viable cells at 72 h of exposure, as observed in the MTT assay.

3.4. Increase in GST activity in HepG2 cells

In agreement with the effects on mRNA and protein levels, an increase in GST total catalytic activity was observed. Compared to the control, CP treatment increased GST activity in a dose-dependent manner (Fig. 6A). Upon treatment with 8 μM CP, a maximum increase of 2-fold was observed at 24 h. At 48 h, the effect was reduced for all concentrations, except 8 μM (Fig. 6B). Treatment with MeP produced similar effects in the total GST activity (Fig. 7A and B). A maximum increase of 1.2-fold was observed at 48 h upon treatment with 6 μM MeP (Fig. 7B).

4. Discussion

hGSTA1/A2 induction occurs in response to diet, therapeutic drugs and other xenobiotics; however, the range of inducers explicitly studied in humans is limited compared to those of mouse and rat enzymes (Morel et al., 1994; Hayes and Pulford, 1995;

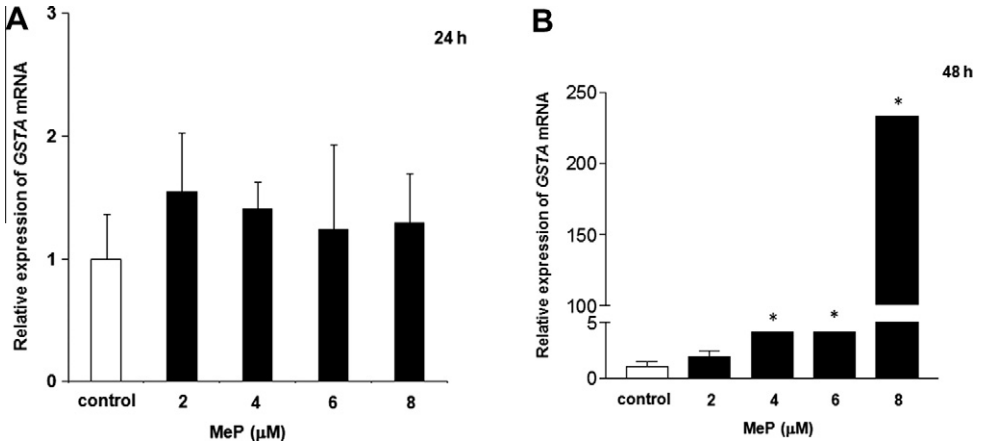

Fig. 3. Effects of methyl parathion (MeP) on GSTA1 mRNA levels in HepG2 cells. HepG2 cells were treated with CP (2–8 μM) or 0.05% of DMSO (control). After 24 h (A) or 48 h (B) treatment, the relative expression of GSTA mRNA was determined by rtPCR. Changes in the level of expression were determined by normalizing the data to 18S and were expressed relative to the control. Data are given as the mean ± S.D. from two experiments performed in triplicate (*$P < 0.05$ vs. control).

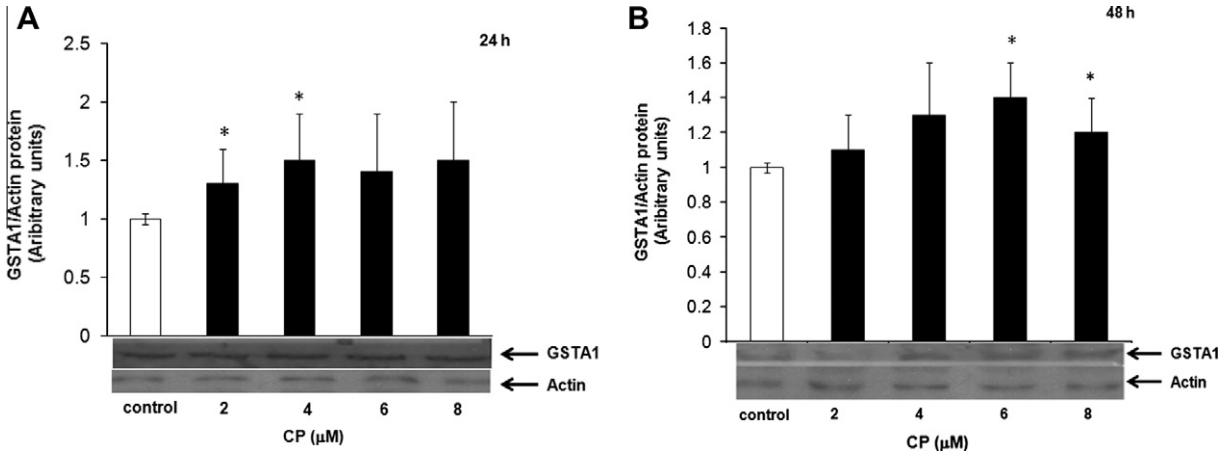

Fig. 4. Effects of chlorpyrifos (CP) on GSTA1 protein levels. HepG2 confluent cultures were treated with various concentrations of CP for 24 h (A) or 48 h (B), and the total cellular extracts were subjected to western blot analysis. The intensities were normalized to Actin and were expressed relative to the control. The relative intensity is given as the mean ± S.D. from three independent experiments. A representative image of 24 and 48 h treatments is shown (*$P < 0.05$ vs. control).

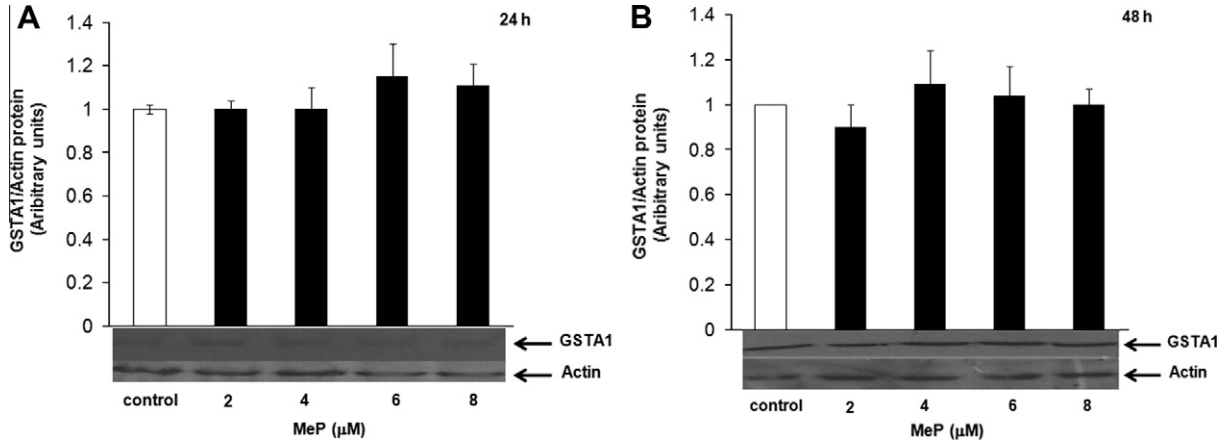

Fig. 5. Effects of methyl parathion (MeP) on GSTA1 protein levels. HepG2 confluent cultures were treated with various concentrations of MeP for 24 h (A) or 48 h (B), and the total cellular extracts were subjected to western blot analysis. The intensities were normalized to Actin and were expressed relative to the control. The relative intensity is given as the mean ± S.D. from three independent experiments. A representative image of 24 and 48 h treatments is shown (*$P < 0.05$ vs. control).

Falkner et al., 2001). Transcriptional activation of GST by various xenobiotics remains an active area of research due to their role in detoxification reactions. The present investigation is the first study on liver cancer HepG2 cell lines that provides evidence that CP and MeP modifies GSTA1 gene expression. Our results showed that CP and MeP increase GSTA1 mRNA, protein and activity levels.

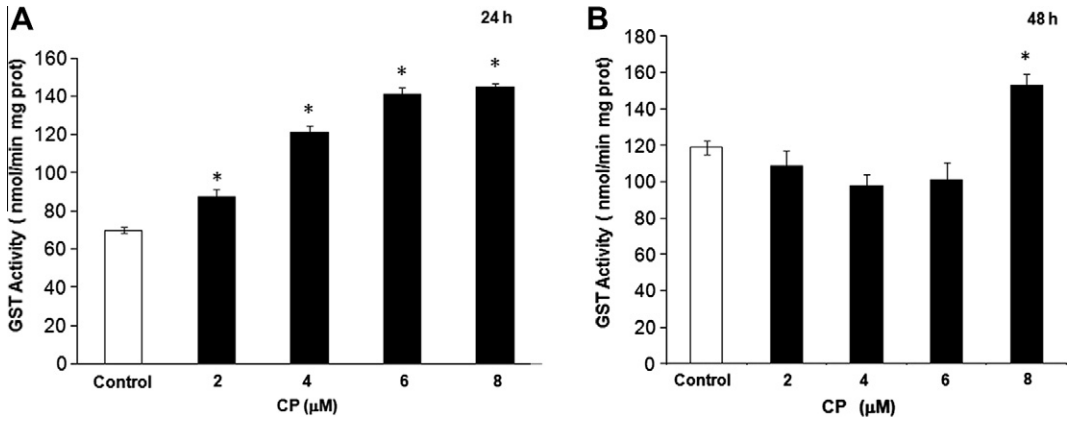

Fig. 6. GST enzymatic activity after chlorpyrifos (CP) treatment. HepG2 cells were treated with various concentrations of CP for 24 h (A) and 48 h (B). The GST activity was determined using GST Assay Kit. Data are given as the mean ± S.D. from two experiments performed in triplicate (*$P < 0.05$ vs. control).

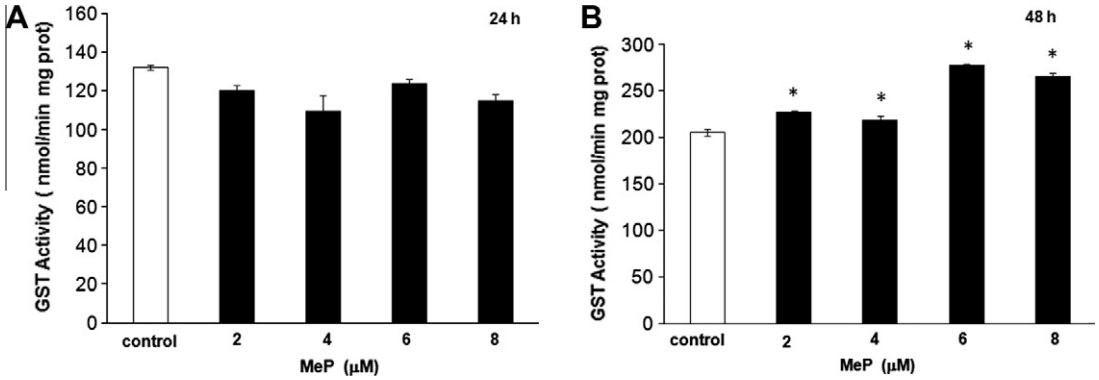

Fig. 7. GST enzymatic activity after methyl parathion (MeP) treatment. HepG2 cells were treated with various concentrations of MeP for 24 h (A) and 48 h (B). The GST activity was determined using a GST Assay Kit. Data are given as the mean ± S.D. from two experiments performed in triplicate (*$P < 0.05$ vs. control).

In addition, there was no correspondence in the magnitude of induction between GSTA1 mRNA and protein levels.

The effects of some pesticides are usually explained by their affinity for nuclear receptors (Coumoul et al., 2002; Lemaire et al., 2004). For example, previous studies have demonstrated that chlordane, dieldrin and chlorpyrifosare able to induce PXR through cytochrome P450 isoforms CYP3A4 and CYP2B6 (Coumoul et al., 2002; Lemaire et al., 2004). Moreover, recent studies demonstrated that PXR overexpression in HepG2 cells increased GST activity (Naspinski et al., 2008). The mechanism of action by which OPs increase or modulate the expression of GST is unknown. Although the present data do not reveal the mechanism by which CP and MeP induce GSTA1 mRNA transcription, the role of PXR in this process is currently under investigation.

Alternatively, CP and MeP effects may be mediated by the Nrf2 transcription factor. GSTs are up-regulated by electrophiles through Nrf2 via the cis-acting antioxidant response element (also called the electrophile response element) (Kang et al., 2000, 2001). In addition, OPs have been shown to induce oxidative stress (Karami-Mohajeri and Abdollahi, 2010; Ray et al., 2010). Furthermore, previous studies have shown that GSH deficiency and exposure of cells to reactive oxygen species (ROS) (e.g., peroxynitrite) or prooxidants (e.g., phenolic antioxidants) enhanced the induction of GST (Kang et al., 2000, 2001, 2002).

Several studies have demonstrated that many of the effects of OPs are due to its interaction with cellular proteins (Calore et al., 1999; Ray et al., 2010). Thus, protein damage produced by OPs may result in protein degradation by the polyubiquitin pathway, which is regulated by a finely tuned balance of ubiquitination and deubiquitination processes (Calore et al., 1999). Alternatively, CP may modify GSTA protein levels by affecting post-transcriptional mechanisms. In previous studies conducted on rats, CP affected the transcription and translation of some genes; these effects were dependent on the dosage (Ray et al., 2010). Therefore, both mechanisms may explain the lack of correspondence between mRNA and GSTA1 protein levels. However, CP and MeP increased GSTA1 mRNA expression in a pattern consistent with the observed increase in total GST activity. Studies on human liver tissue and recombinant GST isoforms demonstrated that GSTA1 and GSTT1 are responsible for the *O*-demethylation of MeP (Abel et al., 2004), suggesting that MeP induces its own metabolism.

The potential inductive effects of OPs on GSTA1 could result in therapeutic failure or toxicity modulation by endobiotics and xenobiotics that are metabolized by GSTA1. Furthermore, polymorphism in GSTA genes could result in reduced enzymatic activity and detoxification capacity and could confer a higher response to chemotherapeutic agents due to reduced degradation (Ekhart et al., 2009). Although the results obtained in the present study indicate that CP and MeP modify GSTA1 expression, this may not reflect accurately what would happen in human livers or fresh isolated hepatocytes, since substantial differences were observed in term of enzyme expression, like P450, between *in vitro* and *in vivo* systems (Sumida et al., 2000; Gimelbrant et al., 2007; Plagnol et al., 2008; Lin et al., 2011). Therefore, we expect alterations in GST-dependent xenobiotic metabolism at lower pesticide concentrations.

GSTA activity continues to be investigated due to the abundance of this enzyme in the liver and its role in the biotransformation of a large number of substrates with diverse chemical structures. Further investigations have to be done in order to elucidate the effect of OPs on GSTA1 in humans, as well as the molecular mechanism through which xenobiotics positively or negatively regulate the expression of these enzymes. In conclusion, HepG2 cell cultures treated with CP and MeP could be a useful model for studying GSTA1 and its role in the metabolism of xenobiotics in the liver.

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