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RESEARCH

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Irreversible methadone-induced *GSTP1* downregulation in SH-SY5Y cells

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Abstract

Background Methadone has been reported to downregulate the expression of glutathione S-transferase P1 (*GSTP1*) among nine antioxidant genes in SH-SY5Y cells after both short- and long-term treatment. GSTP1 plays a key role in the detoxification of many xenobiotics and is frequently associated with various diseases, especially tumors. The objective of this study is to determine whether this change is reversible.

Methods Two different treatment protocols were used. The first protocol evaluated the reversibility of the *GSTP1* mRNA change, while the second protocol evaluated the methylation status of the *GSTP1* promoter site. To investigate the reversibility of the *GSTP1* mRNA change, SH-SY5Y cells were treated with methadone. The drug was then removed from the medium and the cells were cultured in methadone-free medium for a period of time. *GSTP1* mRNA levels were expressed as cycle threshold (*Ct*) values using TATA box-binding protein as a calibrator gene. Methylation at the promoter site was detected by bisulfite treatment.

Results The analysis of variance revealed no significant change in *GSTP1* mRNA levels in the cells after methadone was removed from the medium of methadone-treated cells. The study also examined the methylation status of a CpG island in the promoter of *GSTP1* in the treated cells. The results demonstrate that although methadone downregulates the mRNA level of *GSTP1* in treated cells, it does not induce methylation in the *GSTP1* promoter region.

Conclusions The expression of the *GSTP1* remains downregulated even after methadone removal from SH-SY5Y cell culture medium; however, methylation of the *GSTP1* promoter site does not play a role in this process.

Keywords GSTP1, Methylation, Methadone, SH-SY5Y

Background

Methadone maintenance treatment (MMT) is a proven harm reduction approach for the treatment of drug dependence. Methadone is a potent inducer of NADH/NADPH-mediated reactive oxygen species (ROS) synthesis [1–3]. The deleterious effects of ROS can be prevented by antioxidant systems, particularly glutathione S-transferases (GSTs) [4]. GSTs are classified into several classes, including alpha, mu, kappa, pi, and omega. *GSTP1* (MIM: 134,660) plays an important role in the detoxification of various xenobiotics [4, 5].

The mRNA levels of nine antioxidant genes in SH-SY5Y cells were evaluated following treatment with varying concentrations of methadone (1, 2.5, 5, 7.5, 10, and 20 μM) for different durations (1, 24, and 72 h as short-term treatment and 18 days as a long-term treatment) [6, 7]. The genes were classified into three groups based on the changes in their mRNA levels: (I) Six genes (*CAT*, *SOD1*, *SOD2*, *NQO1*, *NQO2*, and *GSTM2*) showed an increase at 1 h and 24 h (particularly at relatively high methadone concentrations), followed by a decrease at 72 h. (II) The expression levels of two genes (*GSTM3* and *GSTO1*) increased at 1 h, decreased at 24 h, and then increased again at 72 h. (III) *GSTP1* expression levels

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decreased in treated cells from 1 h to 3 days [6]. Among nine antioxidant genes, only the expression of *GSTP1* showed a decrease after short- and long-term methadone treatment in SH-SY5Y cells [6, 7]. However, the reversibility of *GSTP1* downregulation after methadone exposure of SH-SY5Y cells was not investigated. Therefore, the present study was conducted to determine whether this change is reversible.

Epigenetic alterations, such as promoter hypermethylation, are important and often involved in tumor initiation, progression, and recurrence [8–10]. DNA methylation in the promoter of *GSTP1* has been frequently reported in a variety of tumors, including neuroblastoma [11], hepatocellular carcinoma [12], endometrial [13], breast [14], and prostate cancers [15]. Methylation status is involved in the regulation of gene expression. Methadone induces hypermethylation in human DNA [16, 17] and in a number of genes such as the *OPRM1* gene [18, 19]. Therefore, we decided to examine the methylation status of the *GSTP1* gene promoter to determine whether changes in gene expression are irreversible after methadone is removed from the culture medium.

In summary, the present study had two aims: (1) to investigate the reversibility of *GSTP1* downregulation after methadone exposure of SH-SY5Y cells and (2) to determine the methylation status of the *GSTP1* gene promoter in methadone-treated SH-SY5Y cells.

Material and methods

Cell treatments

The human SH-SY5Y cell line has been widely used as an in vitro model in experimental neurological studies, including the analysis of neuronal differentiation, metabolism, neurotoxicity, neuroprotection, and neuronal differentiation [20]. The SH-SY5Y cells have also been used in some experiments to determine the effect of methadone on the expression levels of several antioxidant genes [6, 7]. In the present study, the human SH-SY5Y cell line was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, NCBI: C611). SH-SY5Y cells were maintained in medium (DMEM F-12; 1:1) supplemented with Glutamax, 10% FBS (GIBCO), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma).

Two different treatment protocols were performed. The first protocol was used to test the reversibility of the downregulation of *GSTP1*, and the second protocol was used to investigate the methylation status of the *GSTP1* promoter region. In the first treatment protocol, cells were exposed to methadone at final concentrations of 2.5 and 10 μM for 24 and 72 h and 18 days. The final methadone concentrations and exposure times which chosen for the present experiments were used in previous studies [6, 7]. To determine the reversibility of the decrease in mRNA levels, methadone was removed from the medium and cells were cultured in methadone-free medium. After a period of time, the cells were harvested and the *GSTP1* mRNA levels were measured. In the second treatment protocol, cells were exposed to methadone at a final concentration of 10 μM for 1, 24 and 72 h and 18 days. Cells were harvested, genomic DNA was extracted, and DNA methylation status was determined.

Measurement of GSTP1 expression

Total RNA was extracted using the RNX-Plus kit (CinnaGene, Iran) according to the manufacturer's instructions. The RNA samples were used for cDNA synthesis using the PrimeScript RT Regent Kit (Takara, Japan). *GSTP1* mRNA levels were quantified according to the previously described protocol [6]. The following primer pairs for *GSTP1* (as gene of interest) and TATA box-binding protein (*TBP*, MIM: 600,075, as calibrator gene) were designed using Allele ID v7. 8 software: for *GSTP1*: 5′-ACT CAA AGC CTC CTG CCT ATA C-3′ and 5′-GTC CTT CCC ATA GAG CCC AAG-3′ and for *TBP*: 5′-CCC GAA ACG CCG AAT ATA ATC-3′ and 5′-TCT GGA CTG TTC TTC ACT CTT G-3′. The primers were specific for mRNA and did not amplify genomic DNA. The product size was 121 and 134 bp for *GSTP1* and *TBP*, respectively. The efficiency of the real-time PCR was evaluated by serial dilution of a cDNA sample and the above primers. The efficiency was 0.98% and 101% for the *TBP* and *GSTP1* primer sets, respectively. PCRs were performed in 20 μl final volume containing 25 ng cDNA. The PCR was set at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s.

Relative differences in *GSTP1* mRNA levels between groups were expressed as Ct values. These Ct values were first normalized to that of *TBP* in the same sample and then expressed as fold change relative to untreated SH-SY5Y cells (=1.0). Relative mRNA values were calculated using the equation: $2^{-\Delta\Delta Ct}$ [21].

Determination of methylated CpG

Bisulfite treatment was performed according to the previously described protocol [22]. It is important to note that unmethylated cytosine (C) is converted to thymine (T), while methylated cytosine remains as cytosine. Genomic DNA (800 ng) was treated with sodium bisulfite using the

ZYMO RESEARCH Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. All samples were examined in duplicate.

In the first step, primers for amplification of the upstream *GSTP1* CpG island were designed using Allele ID v7.8 software, and amplification (323 bp) was performed using 10 µL RealQ Master Mix Green (2×) and 1 µM of each primer (primer A: 5′-TGG GAA AGA GGG AAA GGC TTC-3′ and primer B: 5′- CTG ATG CTG CGG GTT GG-3′). Amplification consisted of 10 min at 95 °C, 35 cycles of 20 s at 95 °C, 30 s at 61 °C, using Corbett Research RG-6000 Real Time PCR. A second round of amplification (300 bp) was performed as above using 2.5 µl of the product of the first amplification with the nested primers C: 5′-TGG GAA AGA GGG AAA GGT TTT-3′ and D: 5′-ACT AAA AAC TCT AAA CCC CAT CCC-3′. Amplification consisted of 10 min at 95 °C, 40 cycles of 25 s at 94 °C, 30 s at 60 °C. Second round primers were designed using Zymo Research online software (www.zymo re-search.com/tools/bisulfite-primer-seeker). Quality control of the PCR product was performed by agarose gel electrophoresis. Specific bands were isolated and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific™ K0691), followed by sequencing services provided by MacroGen Ltd.

Statistical analysis

GSTP1 mRNA levels data are presented as mean ± SE for three independent biological replicates. One-way analysis of variance (ANOVA) was performed to analyze the effect of methadone and/or drug withdrawal on *GSTP1* mRNA levels. Multifactorial ANOVA was performed to analyze the effect of methadone concentrations, methadone exposure times, and duration of SH-SY5Y cells cultured in methadone-free medium on *GSTP1* mRNA levels. SPSS statistical package (version 25) was used for data analysis (SPSS Inc., Chicago, IL, USA). A probability of $P < 0.05$ was considered statistically significant.

Results

The present study consisted of two steps. As mentioned earlier, treatment of SH-SY5Y cells with methadone caused a downregulation of *GSTP1* mRNA levels [6, 7]. Therefore, in the first step, we investigated the reversibility of this change by treating the cells with methadone, removing the drug from the medium, and culturing the cells in methadone-free medium for a period of time. Figure 1 shows the *GSTP1* mRNA levels in these cells and their comparison with treated and untreated SH-SY5Y cells. The expression level of the *GSTP1* gene showed a significant decrease at 1, 3 and 18 days after treatment of SH-SY5Y cells with 2.5 and 10 µM final concentration of methadone ($P < 0.001$), confirming the results of the previous studies [6, 7].

To determine the reversibility of *GSTP1* downregulation, cells were exposed to 2.5 µM methadone for 24 h, then methadone was removed from the medium and the cells were cultured in methadone-free medium for 1 and 24 h. Statistical comparison using one-way ANOVA showed that the mRNA level of *GSTP1* in SH-SY5Y cells treated with methadone (2.5 µM) and the cells treated with methadone and then cultured in methadone-free medium were statistically equal to each other ($F = 1.509$; $df = 2, 6$; $P = 0.294$), indicating that the *GSTP1* mRNA level did not return to its normal level when the cells were cultured in methadone-free medium for 1 or 24 h. This means that the mean mRNA levels of *GSTP1* were statistically equal in the groups studied. The same comparisons were made for 5 other sets of experiments and the same results were obtained. There was no significant change in *GSTP1* mRNA levels after methadone was removed from the medium. The results of the statistical tests are shown in Fig. 1.

Further statistical analysis was performed on all datasets using factorial analysis of variance with methadone concentrations, methadone exposure times, and duration of SH-SY5Y cells cultured in methadone-free medium as predictive factors. Statistical analysis revealed that methadone concentrations ($F = 0.343$; $df = 1, 46$; $P = 0.561$), methadone exposure times ($F = 1.953$; $df = 2, 46$; $P = 0.153$), and duration of cells cultured in methadone-free medium ($F = 1.403$; $df = 3, 46$; $P = 0.248$) had no significant effect on *GSTP1* mRNA levels (Table 1). This means that the downregulation of *GSTP1* was independent of methadone dose and methadone exposure time.

In the second step, we evaluated the methylation status of CpG islands in the promoter region of the *GSTP1* gene in SH-SY5Y cells after they were stabilized with methadone treatment for 1, 24, and 72 h. We analyzed a total of thirty-eight CpG dinucleotides, consisting of 28 CpG sites located in the negative (−) region of the gene and ten CpG sites located in the positive (+) region (see Fig. 2A). Interestingly, the results showed that methadone treatment does not induce methylation at any of the CpG sites within the promoter region (Fig. 2B and C). A G to T substitution in the +72 region of the *GSTP1* promoter was observed during sequencing in SH-SY5Y cells.

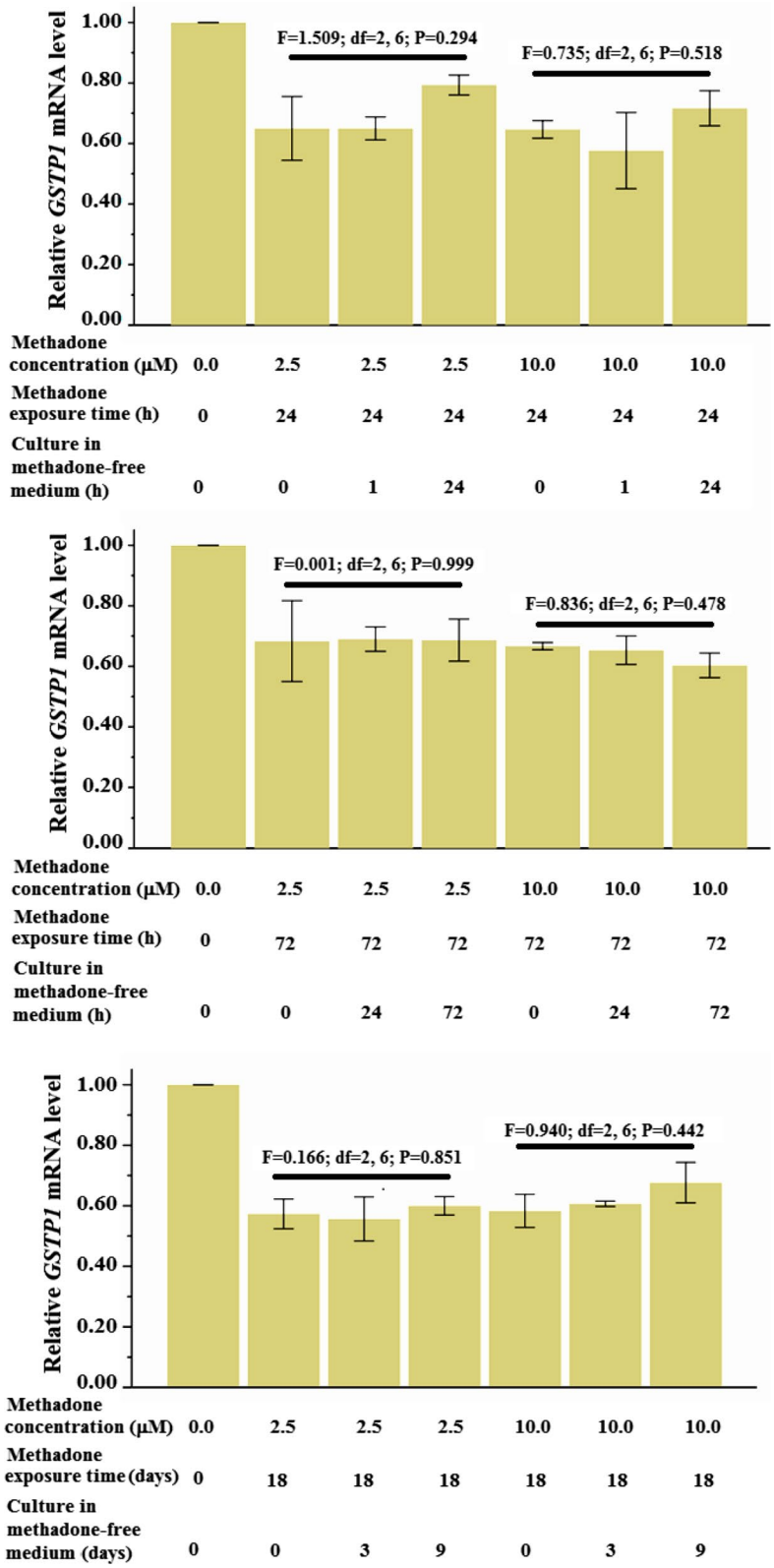

Fig. 1 Expression of *GSTP1* (mean ± SE) in SH-SH5Y cells exposed to methadone and subsequently cultured in methadone-free medium. The results of the analysis of variance are shown above each set of experiments

Table 1 Effect of methadone concentrations, methadone exposure times, and duration of SH-SY5Y cells cultured in methadone-free medium on the *GSTP1* mRNA level

Source of variation	Sum of square	*df*	Mean square	*F*	*P*
Corrected model	0.125*	7	0.018	1.566	0.170
Intercept	18.280	1	18.280	1601.9	< 0.001
Methadone concentrations	0.004	1	0.004	0.343	0.561
Methadone exposure times	0.045	2	0.022	1.953	0.153
Duration of SH-SY5Y cells cultured in methadone-free medium	0.064	4	0.016	1.403	0.248
Error	0.525	46	0.011		
Total	23.128	54			
Corrected total	0.650	53			

*Adjusted *R* square was equal to 0.070

Discussion

Previously, the expression of *GSTP1* was shown to be downregulated among nine antioxidant genes in SH-SY5Y cells after both short-term and long-term treatment [6, 7]. In the present study, we showed no significant change in *GSTP1* mRNA levels in SH-SY5Y cells after methadone was removed from the medium of methadone-treated cells (Fig. 1).

Studies have shown that methylation status is involved in the regulation of gene expression [8–15]. Studies have shown that the promoter region is an epigenetic sensor of synthetic and non-synthetic opioid drugs. The CpG-rich promoter harbors a CpG island, and several studies in different populations have demonstrated higher DNA methylation at this site in opioid addicts and methadone-maintained heroin addicts [23, 24]. Methadone has also been reported to induce hypermethylation in human DNA [16, 17] and in a number of genes such as the *OPRM1* gene [18, 19]. Considering that the downregulation of *GSTP1* in methadone-treated cells is an irreversible change, we hypothesized that *GSTP1* might be hypermethylated by methadone treatment. However, our results showed that although *GSTP1* is downregulated by methadone treatment and the transcript of *GSTP1* remains downregulated after drug withdrawal, methadone treatment does not induce methylation at any of the CpG sites within the *GSTP1* promoter region.

It is not possible to be certain that *GSTP1* mRNA levels will not return to normal later in time. It is likely that the recovery process in the cells cultured in methadone-free medium is very slow and more time is needed for *GSTP1* mRNA levels to return to its normal level. Further experiments are needed to make a final conclusion, including investigation of the change in protein level and enzyme activity of *GSTP1* in the SH-SY5Y cells treated with methadone.

Conclusion

Previous studies have shown that the expression of *GSTP1* is downregulated after both short-term and long-term methadone treatment. The current study showed that methadone treatment irreversibly decreased the mRNA levels in SH-SY5Y cells and that the downregulation of *GSTP1* was not related to methylation by methadone treatment. Further experiments are needed to fully characterize the downregulation of *GSTP1* in methadone-treated cells.

Fig. 2 **A** NCBI reference sequence for promoter region of the *GSTP*1, **B** The *GSTP1* promoter region in SH-SY5Y cells, **C** The *GSTP1* promoter sequence in SH-SY5Y cells after treatment with bisulfite. The red color shows a G > T substitution at the +72 region in the SH-SY5Y cells

Abbreviations

CAT	Catalase
Ct	Cycle threshold
FBS	Fetal calf serum
GSTM2	Glutathione S-transferase M2
GSTM2	Glutathione S-transferase M3
GSTO1	Glutathione S-transferase O1
GSTP1	Glutathione S-transferase P1
MIM	Mendelian inheritance in man
NQO1	NAD(P)H dehydrogenase, Quinone 1
NQO2	NAD(P)H dehydrogenase, Quinone 2
OPRM1	Opioid receptor, MU-1
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
SE	Standard error
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
TBP	TATA box-binding protein

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Author contributions
All authors contributed to the study conception and design. The study conception and design and data analysis were performed by MS. Material preparation, data collection, and measurement of gene expression were performed by KS. All authors read and approved the final manuscript.

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Availability of data and materials
All data are presented in the text.

Declarations

Ethics approval and consent to participate
Study had been accepted by Ethics Committee of Shiraz University (DB-SU-909402; 13.10.2015).

Consent for publication
None.

Competing interests
The author declare that they have no competing interests.

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