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Down-regulation of antioxidant genes in human SH-SY5Y cells after treatment with morphine

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ABSTRACT

Aims: Morphine strongly induces reactive oxygen species (ROS). The deleterious actions of morphine can be countered by antioxidant system. In the present study, we investigated the expression levels of nine antioxidant genes in human SH-SY5Y cells treated with morphine.
Main methods: The cells were treated with three final concentrations of morphine (1, 5, and 10 μmol) for four exposure times (1 h, 24 h, 72 h and 18 days). The mRNA levels were determined using quantitative real-time RCR.
Key findings: Based on the alterations of mRNA levels, the genes might be categorized into three different groups: In the first group, the mRNA levels of the *CAT*, *SOD1* and *GSTM3* genes were significantly down-regulated in all examined experimental conditions. In the second group, the mRNA levels of *SOD2*, *NQO1*, *GSTM2* and *GSTO1* were initially increased and then decreased. In the third group, the mRNA levels of *NQO2* and *GSTP1*, were initially increased and then reached to the control levels. The number of down-regulated genes were significantly increased as a function of exposure time ($\chi^2 = 7.52$, $P = 0.006$). We investigated the effect of morphine (10 μmol) in the absence and presence of N-acetyl-cysteine (NAC, 1 mmol). The mRNA levels revealed significant differences between cells exposed to morphine and cells co-treated with morphine plus NAC. In cases that morphine increased the level of mRNAs, morphine plus NAC, result in decreased mRNA levels and vice versa.
Significance: These findings suggested that there are different pathways for regulation of antioxidant genes after SH-SY5Y cells exposed to morphine and morphine might act through inducing ROS.

1. Introduction

Morphine has been used as a potent analgesic, but its long-term use is associated with tolerance and dependence. Several studies have been demonstrated that opiates may cause oxidative stress in drug dependent individuals [1–4], and morphine-treated patients [5]. Oxidative stress and subsequently apoptosis in the central nervous system is one of the mechanisms for morphine-induced neurotoxicity [6]. It is reported that in human neuroblastoma SH-SY5Y cells treated with morphine, reactive oxygen species (ROS) are generated in a concentration- and time-dependent manner [7]. A recent study was shown that morphine decreases the mRNA level of mu-opioid receptor via production of ROS [7]. The function of L-type Ca^{2+} channel was regulated by ROS [8]. We know that the mu opioid receptor and L-type Ca^{2+} channel play important roles in drug addiction pathway.

Antioxidant defense mechanisms are responsible for scavenging ROS to prevent the oxidative stress. CAT, SODs, NQOs and GSTs are the key antioxidant enzymes to regulate the oxidative stress. It has been reported that morphine decreases SOD and CAT enzyme activities [1,9,10]. Very recently, we reported a significant association between *GSTM1* and *GSTT1* genetic polymorphisms and susceptibility to drug dependency [11]. Taken together, it might be suggested that the antioxidant genes, play an important role in morphine dependent pathways. However, there is no study on the short-term and long-term effects of morphine on the mRNA levels of antioxidant genes. Considering that previous studies have been shown that the SH-SY5Y cell line is a good model for studying the mechanisms of morphine and dependency toward drugs [12–14], here we investigate the effect(s) of morphine on the expression of nine antioxidant genes (*CAT*, *GSTM2*, *GSTM3*, *GSTP1*, *GSTO1*, *NQO1*, *NQO2*, *SOD1*, and *SOD2* genes) in human SH-SY5Y cells, as a neuronal-like cell model.

2. Materials and methods

Human SH-SY5Y cell line (NCBI: C611) was obtained from National Cell Bank of Iran (the Pasteur Institute of Iran, Tehran). The SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture: F-12 (DMEM F-12 1:1) enriched with GlutaMAX, supplemented with 10% fetal bovine serum (FBS) from GIBCO, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

To measure the cytotoxicity of morphine, SH-SY5Y cells were seeded on 96-well plates (1.5×10^4 cells/well, 100 μL complete medium) and

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were allowed to attach overnight. Then, the cells were treated by different concentrations (1 μmol to 1 mmol) of morphine hydrochloride trihydrate ($C_{17}H_2OClNO_3 \cdot 3H_2O$; Cat# MM0037.00, CAS# 6055-06-7 from LGS standards, Swiss, soluble in water 40 mg/mL) and were incubated for 24 h and 72 h. Subsequently, the cell viability was measured by methylthiazol tetrazolium (MTT) dye reduction assay. After obtaining the absorption (at 570 nm), the percentage of cell growth inhibition was calculated.

For treatment of SH-SY5Y cells by morphine, a number of 2.8×10^6 cells were seeded into 10 cm tissue culture Petri dishes. After 18 h the medium was replaced by morphine-supplemented fresh medium. The experiments were carried out at three final concentrations of morphine hydrochloride (1, 5 and 10 μmol). Treated SH-SY5Y cells were incubated at 37 °C for four exposure times (1 h, 24 h, 72 h and 18 day). For 18 day treatments, cells were passaged after every three days and morphine was immediately added. The control cells were maintained in morphine-free growth medium. It should be noted that the human SHSY5Y cells express the UGT2B7 enzyme which is necessary for converting morphine to morphine-6-glucuronide (M6G) as a main metabolites of morphine [15]. Therefore, it is not necessary to use liver S9 fraction for metabolic activation of morphine. The SH-SY5Y cells also were treated with co-treatment of 10 μmol morphine and 1 mmol N-acetyl-cysteine (NAC; C_5H_9-NO_3S) in the media. N-acetylcysteine (NAC) is the acetylated variant of the amino acid L-cysteine and it is widely used as an antioxidant. All experiments were done in triplicate. This study was approved by the Shiraz University ethics committee.

Quantitative real-time PCR conditions and specific primers were described previously [16]. Relative differences in gene expression between groups were expressed using cycle time (Ct) values. These Ct values were first normalized with that of "TATA box-binding protein" (*TBP*) in the same sample and then expressed as fold changes compared to control (set to 1.0). Relative values of transcripts were calculated using the equation: $2^{-\Delta\Delta Ct}$, where ΔCt is equal to the difference in threshold cycles for target and reference. It should be noted that as reported previously the SH-SY5Y cell line has null genotypes of *GSTT1* and *GSTM1* [16]. Therefore, we failed to investigate the alterations of these genes after cells exposed to morphine.

Data were presented as mean ± SE of three independent experiments. Effects of exposure times and concentrations of morphine on the mRNA levels of the examined genes were investigated using

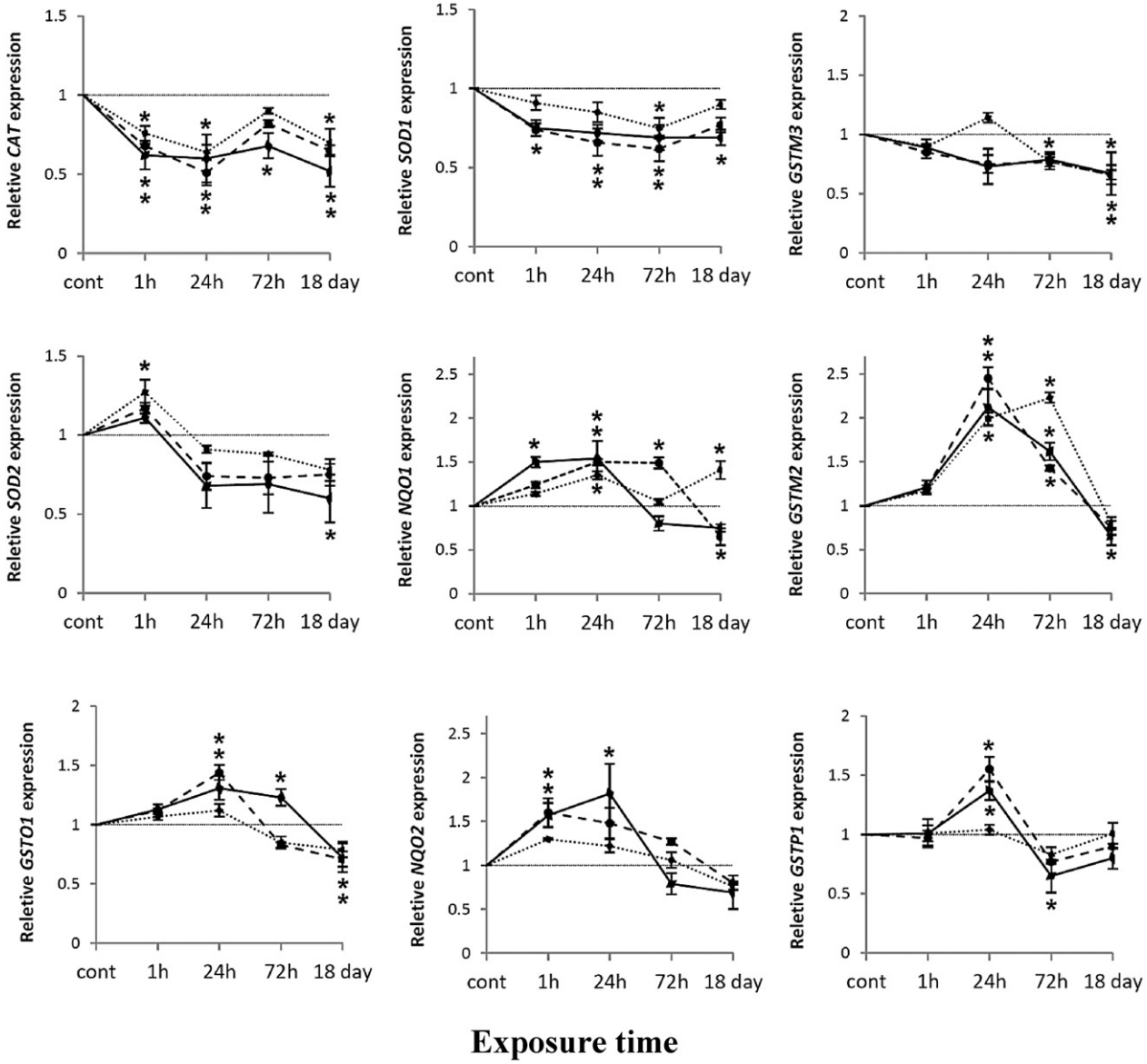

Fig. 1. Relative expression levels of *CAT*, *SOD1*, *SOD2*, *NQO1*, *NQO2*, *GSTM2*, *GSTM3*, *GSTP1* and *GSTO1* in SH-SY5Y cells after exposed to 1 μmol (••••), 5 μmol (—) and 10 μmol (—) morphine at 1 h, 24 h, 72 h and 18 days ($n = 3$, mean ± *SE*). *P < 0.05 all values compared with untreated controls (=1) using Bonferroni post hoc test.