The Effect of Cumene Hydroperoxide on the cAMP Formation in Opiate-dependent Human SH-SY5Y Cells

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Abstract—The human neuroblastoma SH-SY5Y cells were used to assess the effects of cumene hydroperoxide (CHP) on opioid receptor-mediated changes of intracellular cyclic AMP (cAMP). 10μM morphine acted on SH-SY5Y cells significantly inhibited the stimulatory effect of 1μM forskolin (Fs) which caused an increase in the basal level of intracellular cAMP. However, the inhibitory effect of morphine on cAMP accumulation was significantly attenuated when the cells were pretreated with CHP. Chronic activation of μ-opioid receptors by morphine was shown to lead to partial desensitization, upon withdrawal of the opiate agonist, to overshoot in the production of cAMP in SH-SY5Y cells. But CHP pretreatment reduced the stimulatory effect of Fs on cAMP. The SH-SY5Y cells were treated with different concentrations of CHP for 24h, which indicated that CHP was able to cause oxidative damage in SH-SY5Y cells. MDA contents and ROS levels were increased, SOD activation were reduced. We concluded that the function of μ-opioid receptor was significantly decreased. ROS changed the effects of opioid receptor agonists on intracellular cAMP, which associated with adaptive changes in morphine dependence.

Keywords—Opioid receptors; ROS; CHP; cAMP

I. Introduction

Morphine has been widely used for clinical management of chronic pain. Opioid based pharmacotherapy is now the mainstay approach for the management of cancer pain, and has been proven to effectively relieve cancer pain[1]. However, its effectiveness decreases with chronic use, i.e., tolerance develops over time[2]. The prolonged use of opioids is associated with a requirement for ever-increasing doses in order to maintain pain relief at an acceptable and consistent level. This phenomenon is termed analgesic tolerance, as result of cellular adaptations to the presence of the opioids[3], but molecular mechanism of tolerance is still unclear. Management of tolerance and withdrawal symptoms in neonates remains a major challenge.

The risk to health caused by the abuse of opioids has been related to the oxidative stress and free radicals[4,5]. Reactive oxygen species (ROS) generation has been observed in a variety of tumour cell systems following opioid analgesics treatment. Morphine and opioids have been shown to induce oxidative stress in immune system kidneys, epithelial cells and central nervous system (CNS)[6,7]. Previous studies have shown significant depletion of reduced glutathione (GSH) in peripheral organs following acute systemic or central administration of opioids[8]. On the other hand, studies with morphine suggest that NO may play an important role in the development of tolerance to the opiate [9]. Morphine dependence mice showed an increase in NO content in brain.

The free radical theory of aging proposes that deleterious effects of ROS should be responsible for the deterioration of neuronal function[10]. Opioid receptors play a critical role in pain and analgesia. Since free-radical-induced oxidative stress leads to an impairment of neuronal function, it may have a detrimental effect on opioid receptors. The effects of oxidative damage on other receptor systems, such as adrenergic and cholinergic, have been studied[11,12], but the effect of oxidative stress on opioid receptors is poorly understood. It is unclear how oxidative stress contributes to changes in sensitivity to opioid therapy because we do not understand oxidative-stress-induced changes in the opioid system and its receptors and signal transduction pathways.

In the present study, the opioid-responsive differentiated SH-SY5Y neuronal cells were used to investigate acute and chronic opioid action[13]. The SH-SY5Y cells were differentiated to a neuronal phenotype with an increased expression of MOR[14]. CHP is known to be used to induce oxidative stress in both in vitro and in vivo conditions to model neurodegenerative disorders. Cells exposed to CHP resulted in an increase of ROS and the induction of oxidative stress, leading to impaired cellular energy production[15]. We study the effects of exogenous cumene hydroperoxide (CHP) induced oxidative stress on opioid receptor-mediated changes in cyclic AMP (cAMP) in opiate-dependent SH-SY5Y cells.

II. Materials and Methods

A. Materia

Foskolin (Fs), cumene hydroperoxide (CHP), 2,7- dichlorodihydrofluorescein diacetate (DCFH-DA), phosphodiesterase inhibitors, all trans-retinoic acid (RA), dimethyl sulfoxide (DMSO). Morphine sulfate was purchased from Qinghai Technology Co., Ltd.(TD2006-0119). Naloxone was purchased from Beijing Technology Co., Ltd. Beijing, China, 20023762). All other chemicals were of analytical grade and commercially available.

B. Cell culture

The SH-SY5Y neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)
supplemented with 2 mM glutamine, penicillin (20 U/ml), streptomycin (20 mg/ml) (basal DMEM) and 10% (v/v) heat-inactivated Fetal bovine serum (FBS). Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂.

C. Differentiation and drug treatment

At about 65% to 75% confluence, SH-SY5Y cells were differentiated into a neuronal phenotype with RA as described previously[16]. RA (10μM in 0.1% ethanol) was added to the media every other day. In some experiments[15], CHP(0.5,1,2,3μM) was added to the differentiated cells for 24h or CHP (2μM) was added to the differentiated cells for 30min, 2, and 6h. Then either the vehicle or/and 10μM morphine was added into for 10min. Chronic treatment of the cells with morphine was performed as described previously[17]. Incubation periods and drug concentrations were indicated in the text. RA-differentiated SH-SY5Y cells from identical cell passages served as controls.

D. Cell survival assays

Cell survival was assayed by measuring the conversion of the yellow, water soluble tetrazolium salt, MTT to a blue formazan. Cells were plated in 96-well plates (3×10^4 cells/well for cells in 100μl of medium). The cells were exposed to different concentrations of CHP (1-40μM) for 24h. Thereafter, MTT was added at a final concentration of 0.5 mg/ml and incubated for 3h at 37°C. Formazan crystals were dissolved with 150μl DMSO, and optical density was read using the micro-plate reader at 570 nm[18]. Incubation periods and drug concentrations were indicated in the text. RA-differentiated SH-SY5Y cells from identical cell passages served as controls.

E. cAMP accumulation assay

The SH-SY5Y cells were subcultured in 24-well culture plates. Fresh serum-free medium was added for 4–16h before experiments. After drug treatment, extraction of cells was performed. Briefly, the medium was aspirated and the cell monolayer was rinsed with 1ml serum-free medium at 37°C. Subsequently, the cells were incubated for 10 min at 37°C in 0.5 ml/well. DMEM containing 20 mM Hepes (pH 7.4), 0.1 mg/ml BSA, the phosphodiesterase inhibitors IBMX (0.5 mM). Next, 10 μM forskolin (Fs) together with the morphine (acute treatment) were incubated for 10 min at 37°C, after the medium was aspirated and the dishes washed once with ice cold phosphate buffered saline. Chronic morphine treatment was achieved by incubating the cells for 24h with 10μM morphine prior to the addition of Fs. Withdrawal of the chronically applied morphine was achieved either by its removal or the addition of 10μM naltrexone just prior to the 10min assay, or where indicated, by four sequential washes with 0.5ml aliquots of assay medium without IBMX. The two methods of agonist withdrawal resulted in similar effects on cAMP accumulation. The amount of cAMP was obtained following Fs stimulation.

F. Measurement of Intracellular ROS

The ROS generated by CHP were measured with the 2,7-dichlorofluorescin diacetate (DCFH-DA) assay[19]. RA differentiated SH-SY5Y cells were plated at a density of 3×10^5 cells/well in 96-well plates. CHP (0.5,1,2,3μM) was added to the cell medium for 24 h. Following each treatment, intracellular ROS accumulation was monitored after 30min incubation with DCFH-DA (10μM). Intracellular nonfluorescent H₂DCF was oxidized to high fluorescent DCF, which was measured using a fluorescence plate reader (Thermo 3001, USA) (excitation, 485nm, emission: 525nm).

G. SOD activity and MDA assay

SOD activity determination was based on the production of H₂O₂ from xantine by xantine oxidase and reduction of nitroblue tetrazolium. SOD activity assays were done with SOD assay kit according to manufacturer’s instructions. SOD activity was expressed as U/10⁴cells. The MDA content was determined pectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS). The TBARS content was expressed as nmol/10⁴cells.

H. Data analysis

Each set of experiments was repeated at least three times. Data was expressed as mean±S.E.M. (n = 6 wells). The statistical evaluation of data was evaluated by analysis of variance (ANOVA). Values of P<0.05 were considered statistically significant.

III. Results AND DISCUSSION

A. Figures and Tables

1) Effects of CHP on cell survival: The MTT assay was used to assess the effect of CHP on cell viability in SH-SY5Y cells. Different concentrations (1μM, 10μM, 20μM, 30μM, 40μM) of CHP were added to SH-SY5Y cells for 24h, and the cell viability decreased slightly. As shown in (Fig.1), CHP could inhibit the proliferation of SH-SY5Y cells in a dose-dependent manner. The 4.3μM CHP led to a 50% inhibition.

Effect of CHP on cell viability in SH-SY5Y cells

![Figure 1. Effects of CHP on SH-SY5Y cells survival. Differentiated SH-SY5Y cells were cultured for 24 h in basal DMEM without serum or in this medium containing increasing concentrations of CHP. Cell survival was assessed after treatments by the MTT assay.](image-url)

2) cAMP accumulation assay:

The effects of acute, chronic morphine treatments and agonist withdrawal on the Fs-stimulated cAMP levels were shown in (Fig2 ). The effect of acute morphine (10min) on the Fs-stimulated accumulation of intracellular cAMP was
characterized, morphine significantly resulted in low concentrations of cAMP of control (Fig 2). On the one hand, chronic morphine treatment increased Fs-induced intracellular cAMP levels after Fs stimulation. On the other hand, chronic activation (24h) of the μ-opioid receptor followed by agonist withdrawal (obtained by the addition of the antagonist) led to a large increase in the intracellular cAMP levels compared with only a 9.5-fold Fs-induced cAMP increase after vehicle treatment.

In the absence of CHP, morphine acutely resulted in low concentrations of cAMP of Fs treatment group. In cells pretreated for 30min with CHP, Fs increased cAMP from 138.54±4.50 to 281.66±6.00 pmol/mg pro (Fig.3).

In a subsequent series of experiments, dose-response study with CHP was conducted after exposure to 0.5μM to 3μM of CHP for 24h in differentiated SH-SY5Y cells acutely treated with morphine. The cAMP accumulation significantly increased from 147.83±4.58 pmol/mg pro (acute morphine group) to 501.66±5.25 pmol/mg pro (0.5μM CHP pretreatment for 24h) (Fig.4). The results presented in (Fig.3) and (Fig.4) showed that CHP significantly attenuated the inhibitory effect of morphine on intracellular cAMP in SH-SY5Y cells treated with opioid-acutely. CHP showed time-and-dose-dependent increase in the formation of cAMP in SH-SY5Y cells treated with morphine-acutely.

It was compared to show the effects of CHP on cAMP accumulation in SH-SY5Y cells treated by chronic morphine. Chronic treatment with morphine resulted in the accumulation of intracellular CAMP from 1625.32±2.30 (Fs group) to 1786.66±3.71 pmol/mg pro (chronic morphine group) and pretreatments with CHP significantly reduced the accumulation of intracellular CAMP from 1625.32±2.30 (Fs group) to 150.10±4.52 pmol/mg pro (pretreatments with CHP group) (Fig.5). Withdrawal of the chronically applied morphine resulted in 3036.66±3.71 pmol/mg pro accumulation of intracellular CAMP, and pretreatments with CHP significantly reduced the accumulation of intracellular CAMP from 1786.66±3.71 pmol/mg pro (chronic morphine group) to 251.10±4.52 pmol/mg pro (pretreatments with CHP group) (Fig. 6).

Figure 2. The effect of the acute morphine, chronic morphine and naloxone precipitated withdrawal on Fs-stimulated cAMP production in differentiated SH-SY5Y cells. RA-differentiated SH-SY5Y cells were cultured in the presence of morphine (10μM) for 10min, 24h or/and the addition of naloxone(10μM) just prior to the 10 min assay before cells were harvested. Untreated cells of the same passage served as control. Stimulation of cAMP was determined under various conditions as indicated in the text. Data were mean±S.E.M. values from the number of experiments. **p<0.01 vs Fs group.

Figure 3. Effects of CHP on the accumulation of cAMP in acute morphine treatment SH-SY5Y cells. Cells were exposed to 2μM CHP for 30min, 2h, or 6h. Subsequently, cells were treated for 10min with morphine (10μM). Stimulation of cAMP was determined under various conditions as indicated in the text. Each value was the mean±S.D. (n = 3). ** Indicates different from acute morphine treatment, p<0.01 vs acute morphine group.

Figure 4. Effects of CHP on the accumulation of cAMP in acute morphine treatment SH-SY5Y cells. Cells were exposed to different concentrations of CHP for 24h. Subsequently, cells were treated for 10 min with morphine (10μM). The ability of morphine to inhibit Fs-stimulated cAMP accumulation was assessed under various conditions as described in the text. Each value was the mean±S.D. (n = 3). ** Indicates different from acute morphine treatment, P<0.01 vs acute morphine group.
various conditions as indicated in the text. Each value was the mean ± S.D. (n = 3). ** Indicates different from chronic morphine treatment, P < 0.01 vs chronic morphine group.

**Figure 5.** Effects of CHP on the formation of cellular ROS. Intracellular ROS levels were measured by the fluorescent probe DCFH-DA after 24h treatment with CHP or DMSO (vehicle control). The value in the figures represented the relative fluorescent intensity per 10^6 cells determined by fluorescent probe. Data were presented as means ± SEM from three independent experiments. *P<0.05, **P<0.01, vs. vehicle control group.

**Figure 6.** Effects of CHP on the morphone-induced accumulation of cAMP in naloxone withdrawal SH-SY5Y cells. Cells were exposed to different concentrations of CHP for 24h. Subsequently, cells were treated for 24h with morphine (10μM). Fs-stimulated cAMP was determined under various conditions as indicated in the text. Each value was the mean ± S.D. (n = 3). ** Indicates different from chronic morphine treatment, P < 0.01 vs chronic morphine group.

3) Determination of DCFH-DA:

CHP was found to induce the formation of ROS in a wide variety of cells. To determine the levels of ROS after CHP exposure, cellular hydrogen peroxide was measured. As shown in (Fig.3), with an increase in the duration of treatment with 0.5-3μM of CHP, the fluorescence intensity of DCF (generated from DCFH-DA upon oxidation) increased and became significant after 24h compared with untreated cells (Fig.7).

4) Cellular SOD activity and MDA assay:

SOD was one of the most important members of the enzymatic antioxidant defense system and played important roles in the maintenance of cellular redox homeostasis. After exposed to 0.5-3.0μM of CHP for 24h, the cellular SOD level decreased significantly as shown in Table1. Since ROS was responsible for the production of MDA which was used as an index of oxidative damage, the lipid peroxidation level was measured by TBAR assay. Lipid peroxidation level in SH-SY5Y cells treated with CHP increased significantly as indicated by the formation of MDA. Cells treated with 3μM CHP for 24h, the level of MDA was 0.32±0.01 nmol/10^6 cells, which was about 5.1 fold higher than that of untreated cells (0.06 ± 0.01 nmol/10^6 cells).

<table>
<thead>
<tr>
<th>CHP Concentration (μM)</th>
<th>SOD Activity (SOD/10^6 cells)</th>
<th>MDA(nmol/10^6 cells)</th>
</tr>
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<tr>
<td>0</td>
<td>6.749±0.045</td>
<td>0.061±0.004</td>
</tr>
<tr>
<td>0.5</td>
<td>3.421±0.047</td>
<td>0.124±0.015</td>
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<td>0.245±0.010</td>
</tr>
<tr>
<td>3.0</td>
<td>1.284±0.059</td>
<td>0.315±0.012</td>
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</table>

p<0.01 vs control group

B. Discussion

Changes in the effects of opioid receptor agonists on intracellular cAMP were used as a marker of the function of μ-opioid receptors (MOR). Acute exposure of agonist resulted in Fs-induced inhibition of cAMP formation in SH-SY5Y cells.[20] Chronic exposure of differentiated SH-SY5Y cells to morphine led to the rapid development of tolerance. Withdrawal of morphine following chronic treatment (by addition of the antagonist naloxone) led to an immediate increase in cyclase activity (overshoot), resulting in a compensatory up-regulation of cAMP production.[21]. This adaptive response seemed to be a function of the agonist.

CHP one of the major peroxide catalysts was used in pharmaceutical industry, which shown to exert toxic effect on several cells and tissues including peroxidation of membrane fatty acids, early depletion of intracellular ATP and pyridine nucleotides responsible for disruption of the cytoskeleton, impaired DNA repair and mitochondrial collapse, or even induction of early genes leading to apoptosis.[22,23]. Acute treatment with 10μM morphine inhibited the stimulatory effect of Fs. CHP pretreatment resulted in a compensatory up-regulation of adenyl cyclase activity. The inhibitory effect of morphine on cAMP accumulation was significantly attenuated in acutely morphine-treated SH-SY5Y cells. During the course of chronic morphine treatment, morphine increased Fs-induced intracellular cAMP levels in differentiated SH-SY5Y cells, which was consistent with our previous report. And upon withdrawal of the opiate agonist, up-regulation of cAMP
production in the human neuroblastoma SH-SY5Y cells was observed.

The aim of the present study was to investigate the effects of CHP on the function of opioid. Pretreatment with CHP increased intracellular cAMP production in morphine-acutely treated SH-SY5Y cells. But when SH-SY5Y cells pretreated with CHP, there was a significant reduction in intracellular cAMP accumulation in morphine-chronically treated SH-SY5Y cells. Because changes in the effects of opioid receptor agonists on intracellular cAMP were a marker of the function of μ-MOR,[24-26], we concluded that under conditions of lipid peroxidation, the function of MOR was significantly decreased.

We investigated the effect of CHP-mediated intracellular ROS accumulation by using the 2,7-dichlorofluoresce. Findings demonstrated that cells exposed to CHP resulted in a significant increase in intracellular ROS accumulation. Cells were exposed to different concentrations of CHP for 24h, plasma membrane preparations were made and used for the enzymatic antioxidant assay and lipid peroxidation content assay. Findings demonstrated there was a significant reduction in the level of the cellular SOD activity, the lipid peroxidation contents increased in SH-SY5Y cells, indicating that oxidative stress may interact with the effect system associated with the function of opioid receptors, contribute to the impairment of MOR function and change the effect of opioid exposure on μ-opioid receptor signaling.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China (No. 30860074).

REFERENCES