Study on The Effect of Low Concentration of Cinnabar on the Expression of Inflammation Related Genes in HK-2 Cells

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Abstract

Cinnabar, a traditional mineral medicine, as an important ingredient in various remedies has a long history of uses in Chinese traditional medicines. According to Traditional Chinese Medicine (TCM) theory, cinnabar has the functions of sedation, tranquilization and sterilization. However, the specific pharmacological mechanism of cinnabar in the treatment of diseases remains unclear. On the other hand, the medicinal safety of cinnabar remains a major issue because of the high content of toxic heavy metal mercury (Hg) in cinnabar. The aim of this study was to investigate the effect of low concentration cinnabar on the expression of inflammation related genes in cells under stress. Human renal proximal tubular cell line HK-2 cells were treated with 4 nM cinnabar or mercuric chloride (HgCl2) in serum-free medium for 4 days. Following the treatment, transcriptomics studies were carried out based on RNA SEQ technique. At the same time, the total RNA of cells was extracted to verify the mRNA transcript levels of matrix metalloproteinase 1,3,10 (MMP1, 3, 10), interleukin-6 (IL-6) and interleukin-1β (IL-1β). In addition, cells were subjected to the treatment of 100 ng/mL lipopolysaccharide (LPS) to further investigate the inhibitory effects of cinnabar on the expression of inflammation related genes interleukin-6 (IL-6) and interleukin-1β (IL-1β). The results showed that Cinnabar inhibited the mRNA expression of inflammation related genes MMP1, MMP3, MMP10, IL-6 and IL-1β under serum-nutrient starvation. Furthermore, cinnabar exerted robust inhibitory effects on the expressions of IL-6 and IL-1β in cells under dual challenges of nutrient deprivation and treatment of LPS. The findings of the present study suggest that cinnabar has potential anti-inflammatory properties through the inhibition of the expression of inflammation related genes.

Keywords: Cinnabar; Inorganic Mercury; Inflammation; LPS; RNA SEQ

Abbreviations

Hg: Mercury; TCM: Traditional Chinese Medicine; HK-2: Human Renal Proximal Tubular Cell; HgCl2: Mercuric Chloride; MMP1: Matrix Metalloproteinase 1; MMP3: Matrix Metalloproteinase 3; MMP10: Matrix Metalloproteinase 10; RNA SEQ: RNA Sequencing; IL-6: Interleukin-6; IL-1β: Interleukin-1β; LPS: Lipopolysaccharide

Introduction

Cinnabar is a red traditional mineral medicine that is mainly composed of mercuric sulfide (HgS), with a content of 96% [1]. Cinnabar is an indispensable ingredient in many famous Chinese patent medicines, for instance, Angong Niuhuang Wan (AGNHW), cinnabar An-Shen pills and Hua-Feng-Dan (HFD) [2-4].

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It's widely recognized that mercury is a toxic heavy metal element and it mainly exists in the form of inorganic mercury, organic mercury and elemental mercury in nature [5]. However, the toxicity of mercury depends largely on its form of existence, and there's a popular supposition that the toxicity of methylmercury is higher than the inorganic mercury. In addition, different chemical forms of mercury have different injury regions. It has been reported that inorganic mercury mainly damages the kidney system, while organic mercury mainly damages the nervous system [6-8]. The nephrotoxicity of cinnabar has been extensively studied due to the kidney's accumulation of inorganic mercury contained in cinnabar.

A large number of studies have demonstrated that mercury compounds induced inflammation, oxidant stress and apoptosis. It has been shown that HgCl$_2$ provoked inflammatory responses by up-regulating the expression of proinflammatory cytokines and further increased nephrotoxicity [9-11]. There are a lot of differences between cinnabar or cinnabar-containing TCM and other mercury containing compounds in light of toxicity [12]. However, the specific molecular mechanism underlying this difference remains unclear.

Because it involves the reabsorption of a large number of substances, the renal tubular epithelium is vulnerable to the attack of toxicants [13,14]. The proximal renal tubular cells are the main toxic target of inorganic mercury. Inorganic Hg (II) ions can rapidly accumulate in this site and cause acute or chronic renal injury [15]. Mercury will first bond sulfhydryl compounds to form a transportable form entering target cells after mercury is absorbed into the blood due to the high affinity for sulphydryl group [16,17]. Yet, due to the large molecular weight of the protein, it is difficult to transport into the cell after binding with sulphydryl containing protein [18], which will greatly reduce the amount of mercury entering the cell in cinnabar. Human renal proximal tubular cell line HK-2 cells are often used as an effective model for mercury nephrotoxicity study [19]. Therefore, in the present study HK-2 cells were employed to characterize the effect of cinnabar on the effect of inflammation gene expression. Specifically, a serum-free medium culture system was used to avoid protein interference. On the other hand, cinnabar itself is an insoluble compound, so the amount of cinnabar finally entering the cell is extremely low [20]. Hence, a low concentration (4 nM) of cinnabar was chosen in the present study.

Previous reports have shown that deprivation induced stress response and apoptosis, leading to increased susceptibility of cells to toxicity [21,22]. The development of apoptosis and oxidative stress are closely related to inflammation [23]. Therefore, illustrating the effect on inflammation of low concentration cinnabar will provide potential clues to further understanding of pharmacological and toxicological modes of action of cinnabar. Therefore, the effects of low concentration cinnabar on the expression of inflammation related genes was characterized using HK-2 cells.

**Materials and Methods**

**Cells culture**

HK-2 cells were purchased from Chinese Academy of Sciences Kunming Cell Bank. Cells were cultured in (DMEM)/F12 medium supplemented with 10% fetal bovine serum, at 37°C and 5% CO$_2$ in a humidified atmosphere.

**Chemicals and reagents**

Mercuric chloride (HgCl$_2$) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich, USA, and cinnabar (96% HgS) was purchased from Beijing Tong-Ren-Tang Co. (Beijing, China), respectively. Dulbecco's modified Eagle's medium (DMEM)/F12, trypsin-EDTA, fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from GIBCO (Grand Island, NY, USA).

**Preparation of cinnabar solution**

HK-2 cells were purchased from Chinese Academy of Sciences Kunming Cell Bank. Cells were cultured in (DMEM)/F12 medium supplemented with 10% fetal bovine serum, at 37°C and 5% CO$_2$ in a humidified atmosphere. Cinnabar was prepared in DMSO and then supplied.
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with serum free medium and sonicated for 1 hour. The prepared solution was subjected to centrifugation at 12,000 rpm for 10 minutes, and then filtered with 0.22 μm microporous filter membrane. The mercury content of the resultant solution was detected by UPLC-ICP-MS.

Treatments of cells

Resuscitated HK-2 cells were used in experiments after two generations. HK-2 cells (1.5 × 10⁵ cells/well) were seeded in 6-well plates. The cells were divided into 4 groups which were grown in the basic medium, and treated with 4 nM cinnabar in serum-free medium, HgCl₂ in serum-free medium, or 10% FBS, respectively, for 4 days. Upon the completion of the treatment, one part of the cells was collected for RNA sequencing, and the other part of the cells was collected to extract total RNA for the validation the expression of MMP1, MMP3 and MMP10. To examine the effect of cinnabar and HgCl₂ on the gene expressions under LPS, cells were divided into three groups which were treated with basic medium, cinnabar and HgCl₂ with or without the present of 100 ng/ml LPS in serum-free medium, respectively, for 4 days. Following the treatment, total RNA was extracted to determine the expressions of IL-6 and IL-1β.

RNA-sequencing and data processing

After 4 days treatment, cells were harvested, and total RNA was isolated. The total RNA was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). Oligo(dT)-attached magnetic beads were used to purify mRNA. The mRNA was segmented by adding interruption reagent to the mRNA. The first strand of cDNA was synthesized by reverse transcription after the segmentation of mRNA, followed by a second-strand cDNA synthesis. A-Tailing Mix and RNA Index Adapters were added to end repair. Then, the linker was amplified by PCR and the products were heat denatured and circularized by the splint oligo sequence to get final library. The final library was amplified with phi29 to make DNA nanoball (DNB). DNBs were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGiseq500 platform (BGI-Shenzhen, China).

Validation of gene expression by real-time PCR

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was employed to validate the expression level of genes. Total RNA was isolated from all the groups using Trizol reagent (Takara, Dalian, China). Total RNA was quantified by ND-2000 spectrophotometer. cDNA was synthesized using the PrimeScript™II first-strand cDNA synthesis kit (Takara, Dalian, China) with the temperature program: 37°C for 15 minutes, 85°C for 5s. The sequences of the specific sets of primers were as follows: GAPDH up: 5′-CGACCACTTTGTCAAGCTCA-3′; GAPDH down: 5′-AGGGGTCTAATGGCAACTG-3′; MMP1 up: 5′-GGTCTCTGAGGGTCAAGCAG-3′; MMP1 down: 5′-AGTTCATGAGCTGCAACACG-3′; MMP3 up: 5′-GCAGTGCCTATCC-3′; MMP3 down: 5′-GAGTGTCGGAGTCCAGCTTC-3′; MMP10 up: 5′-GGCTCTTTCACTCAGCCAAC-3′; MMP10 down: 5′-TCCCGAGAACAGATTTTG-3′; IL-1β up: 5′-GGGCGAAAAGAATC-3′; IL-1β down: 5′-TTCTGCTTGGAGGTGCTGA-3′; IL-6 up: 5′-TACCCCCAGGAGAAGATCC-3′; IL-6 down: 5′-TTTTCTGTGCGTAGCCCTTTF-3′. Real-time RT-PCR was performed using an SYBR Green Master Mix (Takara, Dalian, China) and the conditions were as follows: pre-denaturing step at 95°C for 3 minute, 95°C for 10 second, and annealing temperature for 45 second. Each sample was analyzed with three duplicates.

Statistical analysis

SPSS 24.0 software was used to analyze the data. One-way analysis of variance and t-test were used for analysis. Data are expressed as mean ± SD (standard deviation). P < 0.05 was considered statistically significant.

Results

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Effect of cinnabar on inflammation related genes based on RNA Seq

RNA-Seq is a novel and Powerful technology for studying transcriptomics to reveal the molecular mechanism of specific biological process and disease occurrence process from the genetic level [24]. The inflammation related genes were selected from the sequencing results and produced a graphical heatmap for showing profile change of genes. These genes were composed roughly of proinflammatory mediators and their receptors (IL-1β, IL-6, IL1R1, TNFCR), intra cellular signal transduction molecules (MYD88, IRAK1, TRAF6), apoptosis related genes (NF-κB, FAS, MAP2K3, GDF15), matrix metalloproteinase (MMP1, MMP3, MMP10) and other genes as shown in figure 1. In contrast to the treatment with HgCl₂, the expression levels of IL-1β, IL-6, TNFSF15, MMP1, MMP3, and MPP10 were significantly down-regulated in the cells treated with cinnabar.

Verification of MMP1, MMP3, and MMP10 by RT-PCR

As shown in figure 2, treatment of cinnabar resulted in significant decrease of the mRNA expression level of MMP1, MMP3, MMP10, compared to HgCl₂ group (P < 0.05), while the transcriptional levels of MMP3, MMP10 were significantly reduced compared with control group (treated with basic medium). A 10% FBS was include to indicate normal cell expression.
Verification of IL-6 and IL-1β by RT-PCR

Four days after treatment of cinnabar and HgCl$_2$ at constant mercury concentration, the expression of IL-6 and IL-1β was determined. As shown in figure 3 the mRNA expression levels of both IL-6 and IL-1β were dramatically decreased (p < 0.05) following treatment with cinnabar compared with HgCl$_2$, suggesting a transcriptional regulation of cinnabar on the expression of IL-6 and IL-1β.

Expression of proinflammatory factor IL-6 and IL-1β under dual challenges of nutrient deprivation and treatment of LPS

The expression level of IL-6 and IL-1β were significantly lower in HK-2 cells exposed to cinnabar (p < 0.05) compared to the cells in the control and HgCl$_2$ treated groups. Moreover, the results showed that the expression of IL-6 was dramatically increased compared to the control-exposed cells under dual challenges of nutrient deprivation and treatment of LPS.

Discussion

Cinnabar (α-HGS) has been applied in traditional Chinese medicine for thousands of years. However, the application of cinnabar and cinnabar containing Chinese patent medicines has been greatly limited because of the safety concerns of the toxicity of its mercury content. In recent years, a large number of studies have shown that cinnabar is not as toxic as other mercury compounds, and its absorption and accumulation in the intestine and kidney are far lower than other mercury compounds [25,26]. Cinnabar has sedative, tranquilizing and bactericidal effects. In addition, it has been reported that Cinnabar containing traditional Chinese medicines such as Angong Niuhuang Wan also possess neuroprotective properties [27]. Cinnabar, as a therapeutic drug, has a very low concentration in the body [28]. Yet, the effect of very low concentration of cinnabar on inflammation related genes has not been reported.

The mercury content in the target organ largely determines the strength of toxicity of a mercury compound [29,30]. However, a large number of in vivo and in vitro studies have found that cinnabar accumulation in target organs and cells is lower than other mercury compounds [31,32]. This suggests that it may be more reasonable to use the same mercury content to dissect the differential toxicity mechanisms between cinnabar and other mercury compounds (experiment with completely dissociated mercury from cinnabar). Therefore, to avoid the chelation of proteins in serum to mercury ions, in the present study we used basic medium to prepare cinnabar and challenge the cells with serum deprivation that induces oxidative stress and increases sensitivity of the cells to toxic attacks [21].

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It has been shown that serum deprivation induced the increase of pro-inflammatory factors such as IL-6 and IL-1β which further induced inflammation [9,33]. According to our experimental results, cinnabar was different from other mercury containing compounds in the induction of inflammation and proinflammatory factors. Cinnabar treatment significantly down-regulated the mRNA expression of IL-6 and IL-1β (As shown in figure 3). When cells were given the additional challenge of 100 ng/mL LPS, it was found that the relative gene expression of IL-6 and IL-1β was still significantly suppressed in the presence of cinnabar, suggesting that cinnabar has anti-inflammatory effects to some extent (Figure 4). However, the specific mechanism needs further study.

Extracellular matrix (ECM) is a kind of macromolecule secreted by cells during their growth and differentiation. It plays an important role in the physiological process of cell migration, differentiation, proliferation, inflammation, apoptosis and other pathological processes. Under pathological conditions, the imbalance of ECM production and degradation leads to a large number of ECM accumulation, which leads to a series of pathological changes at the cellular level [34,35]. What’s more, ECM1 is highly expressed in macrophages, under inflammatory conditions [36]. In our experiment, the expression of ECM in cinnabar group was significantly lower than that in HgCl2 group. Meanwhile, the expression of GDF15 was remarkably down-regulated (Figure 1). Research suggests that heavy metal pollution can also stimulate the expression of MMP, which can lead to inflammation, tissue remodeling, cancer and so on. In addition, mercury exposure increases the activities of MMPs in systemic circulation [37-39]. Consistently, we showed that the relative expression of MMP1, MMP3 and MMP10 was evidently up-regulated by treatment with HgCl2. However, they were significantly down-regulated after incubation with cinnabar (As shown in figure 1 and 2). These findings provide new insight into the biological mechanisms possibly playing a role in the safe use of cinnabar. However, further understanding of the mechanisms remain to be uncovered by more studies.

The mercury content of cinnabar in target cells is very low. Therefore, *in vitro* experiments, it is more reasonable to study the pharmacological effect of cinnabar at low concentration. In our experiment, the expression of pro-inflammatory factors, extracellular matrix and matrix metalloproteinase were significantly reduced in cells treated with cinnabar at the concentration of 4 nM, which suggests that low concentration cinnabar in a certain process may have anti-inflammatory effect.

**Figure 4:** The expression of proinflammatory factor IL-6 and IL-1β in the presence of LPS. Values were calculated from three independent experiments and presented as mean ± SD. * and # indicate significantly different as compared to controls and HgCl2, respectively (p < 0.05).
Conclusion

In the present study we demonstrate that cinnabar has the capability to inhibit the inflammation through regulating the gene expression of pro-inflammatory factors, extracellular matrix and matrix metalloproteinases. Furthermore, cinnabar exerted robust inhibitory effects on the expression of inflammation related genes in HK-2 cells under dual challenges of nutrient deprivation and treatment of LPS.

Declarations of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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