Protective effect of coumarin-pi against t-BHP-induced hepatotoxicity by upregulating antioxidant enzymes via enhanced Nrf2 signaling

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Abstract

Coumarin-pi, a new coumarin derivative isolated from the mushroom *Paxillus involutus*, has antioxidative activity, but the underlying mechanism against intracellular oxidative stress is still unclear. This study investigated its cytoprotective effects and the antioxidative mechanism in tert-butyl hydroperoxide (t-BHP)-induced HepG2 cells. The results demonstrated that coumarin-pi suppressed t-BHP-stimulated cytotoxicity, cell apoptosis, and generation of reactive oxygen species (ROS). Additionally, coumarin-pi promoted nuclear factor erythroid 2-related factor 2 (Nrf2) expression and upregulated the protein expression of antioxidantenzymes, including heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidase (NQO1), glutamyl cysteine ligase catalytic subunit (GCLC), and glutamate-cysteine ligase regulatory subunit (GCLM). After coumarin-pi treatment, transcriptome sequencing and bioinformatic analysis revealed that 256 genes were differentially expressed; interestingly, only 20 genes were downregulated, and the rest of the genes were upregulated. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation were used to identify changes in metabolic pathways. Collectively, the results presented in this study indicate that coumarin-pi has a protective effect against t-BHP-induced cellular damage and oxidative stress.

Keywords Coumarin-pi · HepG2 cells · Oxidative stress · Nrf2 · Transcriptome sequencing

Introduction

Oxidative stress, an imbalance between the oxidative and antioxidative systems of cells and tissues, is a result of the overproduction of oxidative-free radicals and associated reactive oxygen species (ROS) [1]. Oxidative stress plays an important role in the pathogenesis of Alzheimer's disease (AD), and antioxidants may be useful for AD treatment [2, 3]. Oxidative stress can also contribute to atherosclerosis, cancers, diabetes, etc. [4–6].

Cells have elaborated various antioxidant defense mechanisms to scavenge ROS and reduce oxidative stress [7]. The transcription factor Nrf2 which binds DNA antioxidant response elements (AREs) is needed to improve the progression of various diseases, especially those caused by oxidative stress-related transcription factors [8, 9].Under physiological conditions, Nrf2 constitutively binds the linker protein, Kelch-like ECH-associated protein 1 (Keap1, also known as a repressor of Nrf2). When exposed to ROS or electrophilic molecules, Nrf2 detaches from Keap1 and is transferred to the nucleus to activate several genes for antioxidant and detoxification [10], such as heme oxygenase-1 (HO-1), NAD(P)H quinone dehydrogenase 1 (NQO1), glutamate-cysteine ligase modifier (GCLM), and catalytic (GCLC) subunit. Activation of Nrf2 has been investigated as a therapeutic target for the prevention and treatment of liver diseases [11].

Interestingly, numerous drugs, including abundant phytochemicals, can activate the Nrf2 signaling pathway, which

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improves the antioxidative capacity and reduces apoptotic cell death [9]. A new coumarin derivative was isolated from *Paxillus involutus* and named coumarin-pi [12]. The coumarin derivatives were reported to have numerous biological activities, such as anticoagulant, anticancer, antioxidant, antiviral, anti-diabetics, anti-inflammatory, antibacterial, antifungal, and anti-neurodegerative [13, 14].

This molecule displayed potent antioxidant activity against DPPH with an IC_{50} value of 16.3 µg/mL compared with the positive control BHA (59.9 µg/mL) [12]. Consequently, we investigated the cytoprotective effect of coumarin-pi on oxidative stress in t-BHP-induced HepG2 cells.

Extraction and isolation

Paxillus involutus fruit body was isolated from the Yanshan Mountain, Hebei Province and identified by Prof. Li-an Wang. The fresh fruit body was dried in the constant temperature drying box at 42 ℃ until there was no weight change and was powdered in a grinder. 3 kg powder of dried *P. involutus* fruit body was stirred using 30 L of ethyl acetate for 2 h and left to stand overnight at 4 ℃. The remaining precipitate was re-extracted 3–5 times following the protocol described above. The organic layer was evaporated in vacuum yield crude extract (94 g).This residue was separated over silica gel CC by gradient elution with petroleum ether–ethyl acetate (1:0 to 0:1). The fraction eluted by ethyl acetate was evaporated in vacuum and re-separated by silica gel CC with $CHCl_3$–MeOH (1:0 to 0:1). The fraction eluted by $CHCl_3$–MeOH at 10:1 was separated by Sephadex LH-20 (MeOH) to obtain coumarin-pi.

Cell culture

HepG2, HCT116, BGC, and RAW 264.7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM), and K562 cells were cultivated with 1640 medium. These cells were supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL) at 37 °C in 5% CO_2. All the cells were kindly provided by professor Junxia Zhao form Hebei Medical University.

Cell viability assay

Cell viability was evaluated by CCK8 assays. HepG2, HCT116, BGC, RAW 264.7, and K562 cells were first grown in 96-well plates for 24 h. HepG2 cells were plated at 1×10^4 cells/well, and the remaining cell density was 3×10^4 cells/well. Then, the cells mentioned above were individually incubated with coumarin-pi (0–48 µM) for 6 h.

Next, 10 µL of CCK8 was added to each well and incubated for 2 h. Then, the absorbance of CCK8 was measured at 450 nm using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, United states of America).

HepG2 cells were plated in 96-well plates (1×10^4 cells/well) and cultured for 24 h. The cells were pretreated with various concentrations of coumarin-pi (0–48 µM) for 6 h and subjected to t-BHP (400 µM) for 3 h. After the supernatant was discarded, 100 µL of DMEM culture medium and 10 µL of CCK8 solution were added for another 2 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, United states of America).

Measurement of intracellular ROS levels

HepG2 cells were plated in 96-well plates (1×10^4 cells/well) and cultured for 24 h. The cells were pretreated with various concentrations of coumarin-pi (0–48 µM) for 6 h. Then, the cells were treated with 0.4 mM t-BHP for 30 min to generate ROS and stained with 1 µM DCFH-DA for 40 min. Fluorescence was detected on a fluorescence-detecting microplate reader with excitation/emission wavelengths of 485/525 nm, and the cell morphology was observed under a inverted microscope (Leica DM IRB, Leica, Wetzlar, Germany) [15].

Quantification of apoptotic cells and necrotic cells

HepG2 cells were grown in 60×15 mm (1.25×10^6 cells/well) plates for 24 h, treated with various concentrations of coumarin-pi (0–48 µM) for 6 h, and subjected to t-BHP (2 mM) for 1 h. Then, the cells were washed three times with PBS, collected, and centrifuged at $300 \times g$ for 10 min at 4 °C. Subsequently, the cells were stained with Annexin V-FITC and propidium iodide (PI). The percentages of apoptosis and necrosis were determined by flow cytometry (Beckman-Coulter, Brea, CA, USA).

Western blot analysis

HepG2 cells were homogenized and solubilized using RIPA lysis buffer supplemented with 1 mM PMSF for 20 min and then centrifuged for 15 min at 4 °C and 12,000 rpm. Western blot analysis was performed as described by previously published methods [16]. An equal amount of protein was separated on SDS polyacrylamide gels and transferred to PVDF membranes (Millipore). The membranes were blocked with 10% skim milk in TBST buffer for 1 h at room temperature

and probed with the appropriate primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. The protein bands were detected using an enhanced chemiluminescence detection system (Bio-Rad laboratories, Hercules, California, US).

RNA-seq transcriptomic assay

HepG2 cells were grown in 100×20 mm (3.5×10^6 cells) plates for 24 h and treated with 24 μM coumarin-pi for 6 h. The treated cells were harvested and treated by TRIzol® Reagent according to the manufacturer's instructions (Invitrogen) for total RNA extraction. The genomic DNA was removed using DNase I (TaKara). Then RNA quality was determined to construct sequencing library. The untreated HepG2 cells were used as control, and there were three repetitions in both groups. For the mRNA-seq assay, the samples were submitted to Shanghai Majorbio Bio-pharm Technology Corporation for RNA-seq. The method and data analysis process are the same as those reported by Rui [17]. Briefly, Poly(A) mRNA was isolated and then converted to double-stranded cDNA using random hexamer primers (Illumina) through reverse transcription. The synthesized cDNA was modified according to Illumina's library construction protocol and PCR amplified using Phusion DNA polymerase (NEB) for 15 PCR cycles. The products were sequenced with the Illumina HiSeq xten. Functional enrichment analysis (GO and KEGG) was performed to identify which differential expression genes were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected P-value ≤ 0.05 compared with the untreated group.

Data analysis

The data were analyzed using t tests with GraphPad Prism 6.0 software, and statistical significance was defined at $P < 0.05$. All experiments were repeated at least three times, and the data are presented as $\overline{X} \pm SD$.

Results

Effect of coumarin-pi on cell viability

Cells were treated with different concentrations of coumarin-pi (0, 6, 12, 24, 48 μM) for 6 h, and cell viability was determined by CCK8 assays. As shown in Fig. 1, different cells had different sensitivities to coumarin-pi. Coumarin-pi at the tested concentrations did not cause significant cytotoxicity to BGCs (Fig. 1a) or RAW 264.7 cells (Fig. 1b). At 48 μM, this compound had significant cytotoxicity to HepG2 cells ($P < 0.001$) (Fig. 1c). As shown in Fig. 1d and e, K562 and HCT116 cells were more sensitive to coumarin-pi than the other cells. When the concentration reached 24 μM,
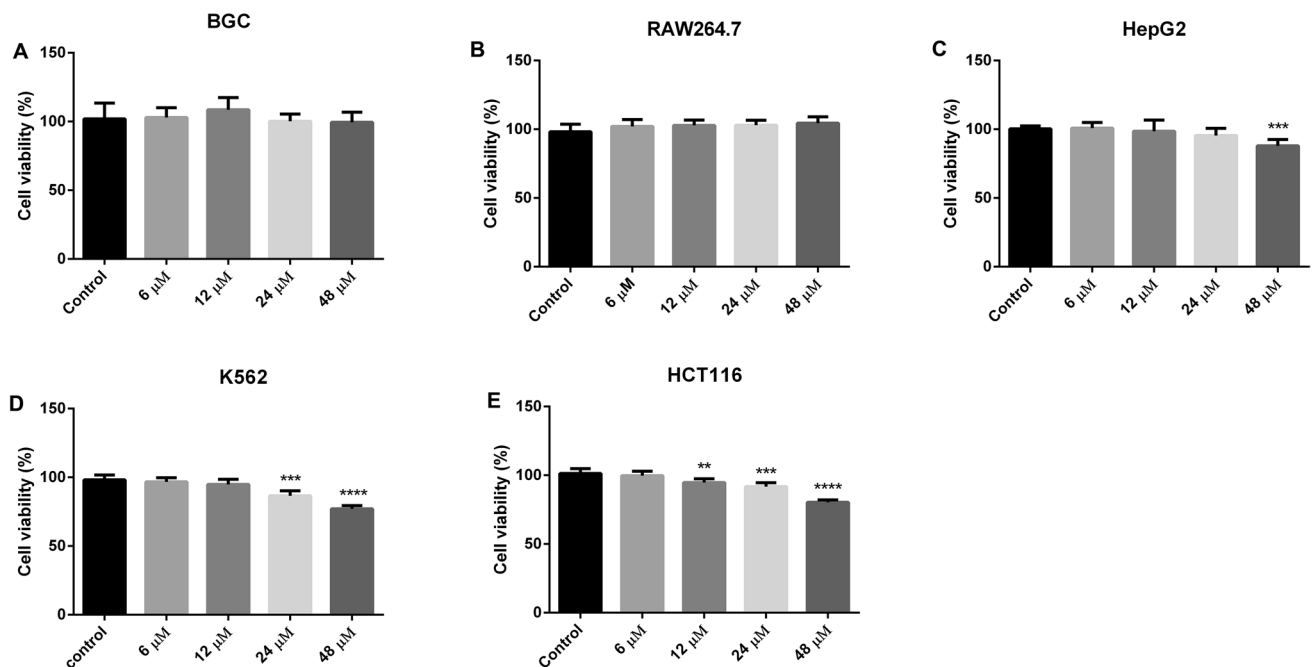

Fig. 1 Effects of coumarin-pi on t-BHP-induced cells. **a–e** Represent BGC, RAW 264.7, HepG2, K562, and HCT116 cells incubated with increasing concentrations of coumarin-pi, and cell viability was determined by CCK8 assays. Values are expressed as the $\overline{X} \pm SD$ ($n = 6$). Different letters indicate statistically significant differences. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. the control group

the activity of the two cell lines decreased significantly ($P < 0.001$) compared with that of the control group.

Coumarin-pi protects HepG2 cells from t-BHP-induced cytotoxicity

Chronic liver diseases are nearly always characterized by increased oxidative stress, regardless of the cause of the liver disorder [18], and HepG2 cells are often used for antioxidant tests. We chose HepG2 cells as the study object.

The cytoprotection of coumarin-pi against t-BHP-induced cell injury was tested. HepG2 cells were pretreated with increasing concentrations of coumarin-pi (6, 12, 24 and 48 μM) for 6 h and then treated with t-BHP (400 μM) for 3 h. As shown in Fig. 2, t-BHP-induced cytotoxicity was restrained by coumarin-pi in a dose-dependent manner. Among the concentrations, 48 μM coumarin-pi significantly restored cell activity to over 72.89%.

Coumarin-pi protects HepG2 cells from t-BHP-induced reductions in ROS production and apoptosis

We studied the effect of coumarin-pi on ROS induced by t-BHP. As shown in Fig. 3, t-BHP can effectively induce ROS overproduction, which increases to approximately 2.5 times compared to that of the control group. After treatment with coumarin-pi, intracellular ROS production was inhibited.

As t-BHP can induce apoptosis, we studied the effect of coumarin-pi on apoptosis in HepG2 cells by using flow cytometry. As shown in Fig. 4a, the apoptosis rate of the
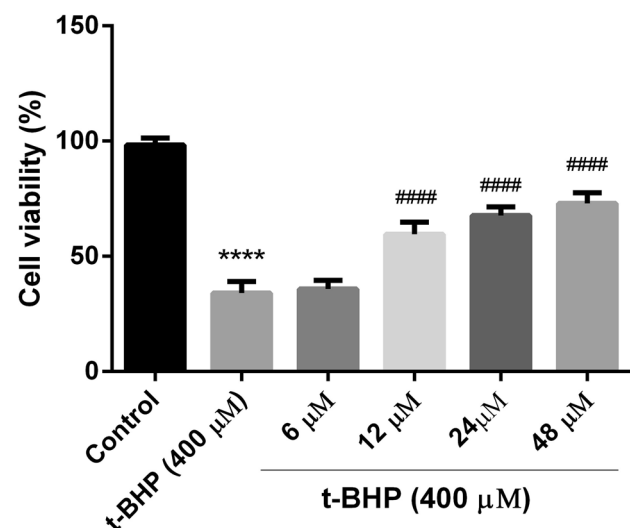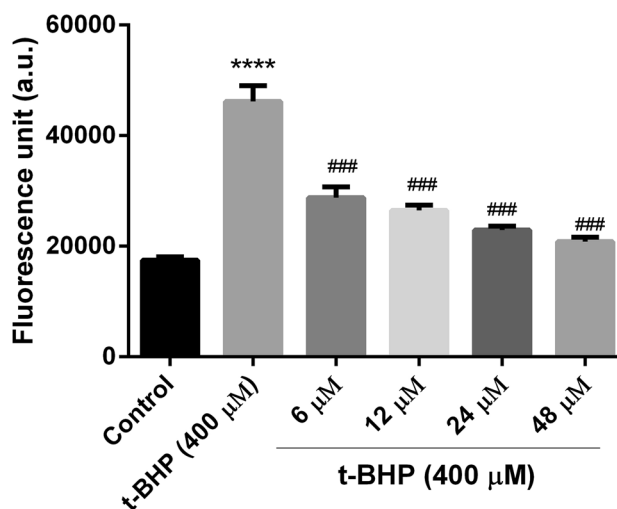

Fig. 3 Effects of coumarin-pi on t-BHP-induced ROS generation. HepG2 cells were pretreated with various concentrations of coumarin-pi (0–48 μM) for 6 h and subsequently subjected to 0.4 mM t-BHP for 30 min to generate ROS and stained with 1 μM DCFH-DA for 40 min

control group was 7.90% and that of the t-BHP group was 46.21%. With increasing coumarin-pi concentrations, the apoptosis rate gradually decreased. When the concentration of coumarin-pi was 48 μM, the apoptosis rate of the cells was close to that of the control group.
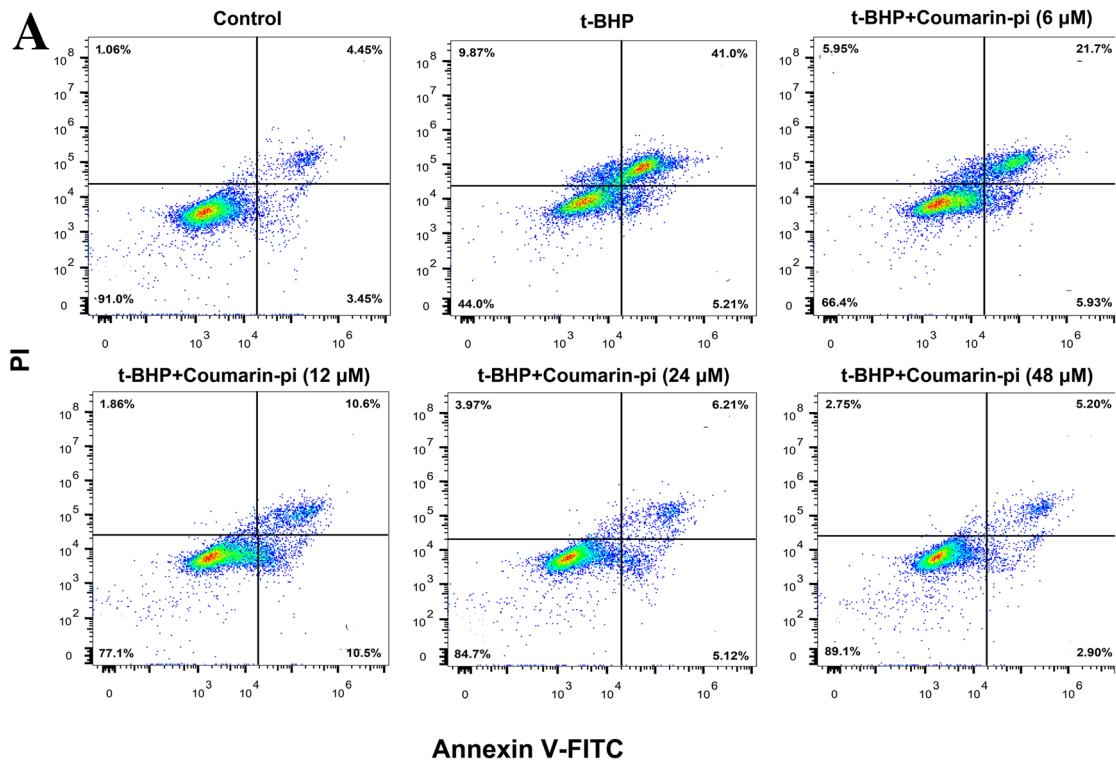
The expression of the members of the caspase family, which have important roles in apoptosis, including caspase-3, caspase-8, and caspase-9, was examined. The expression of cleaved caspase-3, caspase-8, and caspase-9 all increased markedly after treated with t-BHP (Fig. 4b), and their expression decreased gradually when the concentration of coumarin-pi increased.

Our results showed that coumarin-pi could effectively decrease the t-BHP-induced apoptosis and necrosis in HepG2 cells.

Effect of coumarin-pi on the GCLC, GCLM, HO-1, NQO1, and Nrf2 activation induced by t-BHP

Recent reports have suggested that various antioxidative enzymes, including GCLC, GCLM, HO-1, and NQO1, play essential roles in the amelioration of oxidative injury [9]. Consequently, we determined whether coumarin-pi could induce GCLC, GCLM, HO-1, and NQO1 expression to enhance the resistance of cells to oxidative damage. As shown in Fig. 5, coumarin-pi could enhance the expression of these proteins to different degrees.

Nrf2, an essential transcription factor, can translocate into the nucleus and lead to the expression of several of the antioxidant and detoxification genes mentioned above. We tested whether the expression of
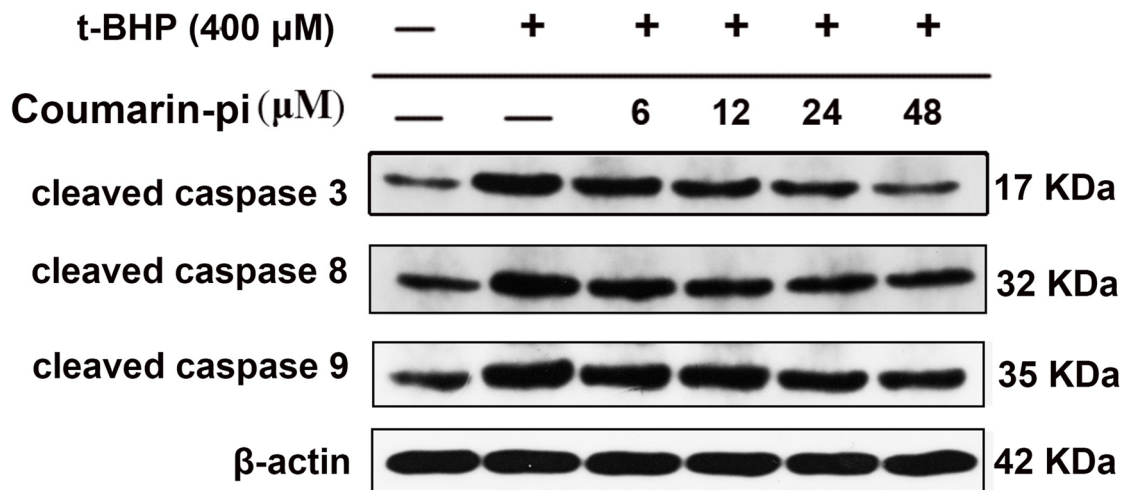

Fig. 2 Coumarin-pi protects HepG2 cells from t-BHP-induced cytotoxicity. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs the control group; ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$ vs the t-BHP group

Fig. 4 HepG2 cells were exposed to various concentrations of coumarin-pi for 6 h and subsequently subjected to t-BHP (2 mM) for 1 h. The percentage of cell apoptosis and necrosis was determined using flow cytometry (**a**) and the expression of cleaved caspase-3, caspase-8, and caspase-9 was detected (**b**)

Nrf2 was upregulated. As shown in Fig. 5, coumarin-pi enhanced the expression of Nrf2. According to the above results, we hypothesized that the Nrf2 signal may play a crucial role against oxidative stress and cytotoxicity.

Analysis of functional enrichment of differentially expressed genes

To further investigate the biological effect of coumarin-pi, we analyzed the transcriptomic changes of the HepG2 cells
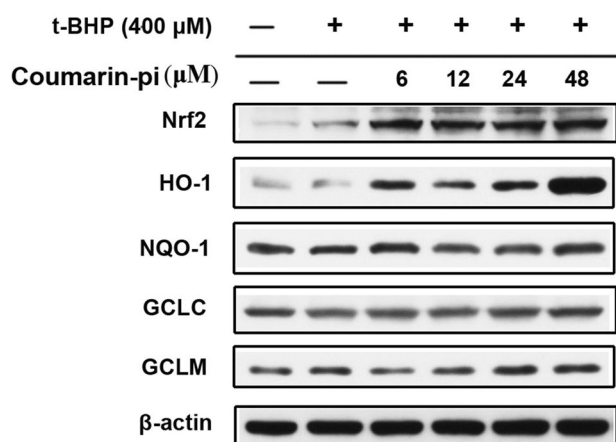
Fig. 5 Coumarin-pi enhanced the protein expression of GCLC, GCLM, HO-1, NQO1, and Nrf2 in HepG2 cells

treated with 24 μM coumarin-pi for 6 h compared with the untreated cells. In the coumarin-pi-treated HepG2 cells, 20 genes were downregulated and 236 genes were upregulated (fold change > 2.0). The differentially expressed genes related to GO terms were enriched in response to stimulus and metabolic process regulation (response to organic substance, cellular response to stimulus, response to chemicals, regulation of cellular metabolic processes, biological regulation, regulation of cellular processes, regulation of metabolic processes, regulation of primary metabolic processes, regulation of biological processes, etc.) (Fig. 6a). The differentially expressed genes related to KEGG pathways were enriched in cancer (microRNAs in cancer, choline

metabolism in cancer, non-small cell lung cancer, endometrial cancer, etc.) (Fig. 6b).

Discussion

This study demonstrated that coumarin-pi can protect HepG2 cells from oxidative stress-induced cell death. In this study, t-BHP, a short-chain analog of lipid peroxide, was chosen as an oxidative stress inducer and could increase intracellular ROS in HepG2 cell lines [19]. Coumarin-pi could alleviate t-BHP-induced cytotoxicity, ROS accumulation, and cell apoptosis. Moreover, this molecule could induce the antioxidant enzyme expression of HO-1, NQO1, GCLC, and GCLM, which are transcriptionally activated by the Nrf2 signaling pathway. It was reported that Nrf2 undergoes nuclear translocation followed by the disaggregation of Nrf2 from the Nrf2-Keap1 complex [20–22]. The change in Keap1 protein needs further study. It was also reported that some antioxidants were associated with Nrf2 activation through the modulation of the Akt and ERK pathways [15, 23, 24]. Whether the protective effect of coumarin-pi is also mediated through these two signaling pathways requires further study. Knockout of Nrf2 signal in HepG2 cell lines is also needed to see if Nrf2 is the key signal of coumarin-pi-induced cytoprotective and antioxidant effects in HepG2 cells.

In addition, we found that some metabolic pathways were regulated in HepG2 cell after coumarin-pi treatment, as shown by transcriptome analysis. In future studies, we will conduct in-depth research on the antioxidant mechanism
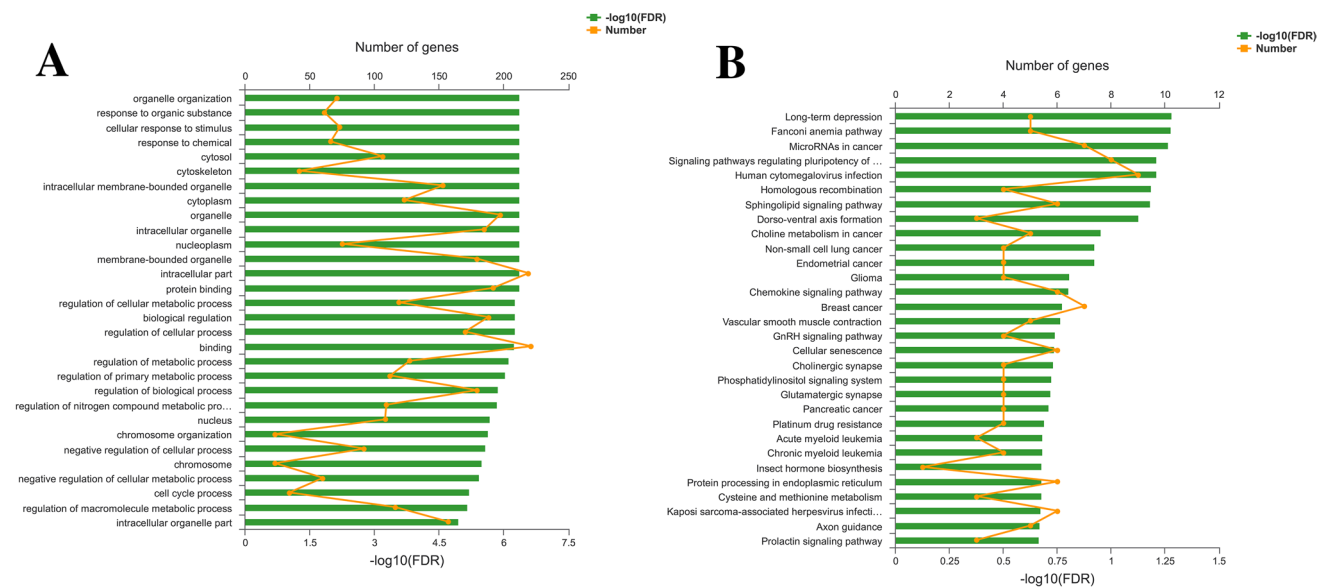

Fig. 6 Enriched Gene Oncology (GO) terms (**a**) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (**b**) of the upregulated genes in coumarin-pi-treated HepG2 cells

of coumarin-pi and further study this compound to determine whether it can be used as a therapeutic or health care product.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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