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# Mechanism of Isoquercitrin Inducing Apoptosis of HepG2 Cells Through PERK-eIF2a-ATF4 Signaling Pathway

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**Abstract** **Original Research Article**

Purpose: To observe the intervention effect of isoquercitrin on related factors in PERK-eIF2a-ATF4 signaling pathway after it acts on HepG2 cells. Materials and Method: HepG2 cells were tested with different concentrations of isoquercitrin. MTT assay was employed to detect the cell proliferation. Expressions of GRP78, PERK, elF2a, ATF4 and CHOP mRNA and protein were measured by qRT-PCR and Western blot. Results: Cell experiments showed that isoquercitrin inhibited the proliferation of HepG2 cells in a dose-and time-dependent manner. Compared with the control group, the expressions of GRP78, PERK, elF2a, ATF4, CHOP mRNA and the expressions of GRP78, p-PERK, elF2a, ATF4, CHOP protein were significantly increased after isoquercitrin treatment, and the expressions of PERK protein decreased significantly. Conclusion: The present study verified that isoquercitrin induce the apoptosis of HepG2 cells, and its mechanism may be related to the intervention of related factors in PERK-eIF2a-ATF4 signaling pathway.

**Keywords:** Isoquercitrin, HepG2, apoptosis, GRP78, CHOP.



## INTRODUCTION

According to the published data of cases of liver cancer in recent years, it is reported that the number of cases of liver cancer may exceed 1 million in 2025, of which 90% are hepatocellular carcinoma, and about 25% have potential mutations [1]. Finding new therapeutic methods and drugs is still a global problem. Isoquercitrin, a member of flavonoids, exists in foods and drinks from herbs, fruits, vegetables and plants. It has higher bioavailability than quercetin, and shows many chemical protective effects in vitro and in vivo, such as antioxidant stress, cancer, cardiovascular diseases, diabetes and allergic reactions [2]. Previous research by our research group found that isoquercitrin can induce cancer cell apoptosis, and discussed its possible mechanism. It was found that isoquercitrin can induce the apoptosis of cancer cells by interfering with endoplasmic reticulum stress pathway of cancer cells [3, 4]. The purpose of this study is to study the effect of isoquercitrin on PERK-eIF2a-ATF4 pathway, one of the three major endoplasmic reticulum stress pathways in HepG2 cells, at the cellular level, so as to provide a new theoretical basis for the study of isoquercitrin in the treatment of liver cancer.

## MATERIALS AND METHODS

### MATERIALS

Human ovarian cancer HepG2 cells were purchased from the cell bank of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. Isoquercitrin was purchased from Spring & autumn Company (purity ≥98%, no. 482-35-9). Antibodies for GRP78 (ab108615), elF2a (ab169528), ATF4 (ab184909), CHOP (ab179823) and β-actin were purchased from Abcam. PERK (D11A8) antibody was purchased from CST Company. P-PERK (DF7576) antibody was purchased from AFFINITY Company. Fluorescent quantitative PCR reagents (no. RR 420A) and TRIzol (no. 9108) were purchased from TaKaRa Company of Japan. The upstream and downstream primers and probes of GRP78, PERK, elF2a, ATF4, CHOP and β-actin were designed and synthesized by InvitrogenTM.

### MTT assay

The logarithmic growth phase of HepG2 cells were inoculated into three 96-well plates at a rate of $2\times10^3$/well for 24 h. Different concentrations of isoquercitrin (0,0.05,0.1,0.2,0.4,0.8 mmol/L) were

added into those 96-well plates, each group was provided with 4 multiple wells, and cultured for 24, 48, 72h. MTT solution was added to each well before testing. After 4 hours, the supernatant from each well was discarded. Subsequently, 100 microliters of dimethyl sulphoxide were added and shaken for 10 minutes. The 96-well plate was placed at 450 nm wavelengths of micro plate reader for detection. The experiment was repeated three times. The experimental results were analyzed according to the formula: the cell growth inhibition rate IR%= (OD drug group-OD control group)/(OD blank group-OD control group)×100%.

**Quantitative PCR assay**

Cells from logarithmic growth phase were inoculated into a 6-well plate with $1\times10^6$ cells per well for 24 h. Different concentrations of isoquercitrin (0,0.1, 0.2, 0.4 mmol/L) were added and collected after continuous culture for 48 h. TRIzol one-step extraction method was used to extract total RNA. Then, total RNA was reversing transcribed for cDNA, and the system of reaction was used for PCR amplification. The primer sequences are shown in Table 1. The reaction system included: sybry was 7. 5μl, $H_2O_2$ was 6. 9μl, cDNA was 0. 2μl and primer was 0. 4μl. The reaction conditions were as follows: the first step was 95℃ for 30 s, then 40 cycles of 95℃ for 5s and 60℃ for 30 s. The results using the $2^{-\Delta\Delta Ct}$ assay to analyze.

**Table 1: The primer sequences of qRT-PCR**

| Gene | Primer Sequences (5'-3') |
|---|---|
| GRP78 | F: GAGAACAGAAACCACCAGC |
| | R: CACGTTGAGAACCTTTGCC |
| elF2a | F: AGGCTGTCTCTAAGCTTTGG |
| | R: TAGTAGTATCTCAAAGCTCGTCC |
| ATF4 | F: ACGATGAGACAGAGTTGCGAC |
| | R: ATCCAAGGCAGCAATTCTCCC |
| PERK | F: CACTGAAGCGACTGATGTCC |
| | R: CACTCCCGCCACAAAGAT |
| CHOP | F: CAGCGACAGAGCCAGAATAA |
| | R: TCAGGTGTGGTGGTGTATGAA |
| β-actin | F: CCACTCCTCCACCTTTGAC |
| | R: ACCCTGTTGCTGTAGCCA |

**Western blot assay**

After 48 h of drug treatment of HepG2 cells, the total protein of the four groups of cells were extracted, and the concentration of protein was determined by BCA method. The protein from each group of cells was taken for SDS-PAGE gel electrophoresis, and the separated protein was transferred to PVDF membrane electrically. Then, the membrane was sealed with sealing solution for 2 h. Appropriate amount of primary antibody (GRP78: 1:20 000, PERK: 1:5 000, p- PERK: 1:5 000, elF2a: 1:20 000, ATF4: 1:20 000, CHOP: 1:20 000, β-actin: 1: 1 000) was added overnight at 4℃. After that, secondary antibody (1: 5,000) was incubated at room temperature for 2 h, and TBST membrane washed 3 times for 10 min each time. Analyze the gray value of the band of image analysis software, and analyze the expression of the proteins of GRP78, PERK, elF2a, ATF4 and CHOP.

**Statistical methods**

The experimental data were analyzed by SPSS25 software. T test was used for comparison between the two groups, and variance analysis was used for comparison among multiple groups. An assessment of $p<0.05$ was considered statistically significant, and all experiments were repeated for 3 times.

## RESULTS

The therapeutic effects of isoquercitrin on HepG2 cells viability

Different doses of isoquercitrin (0,0.05,0.1,0.2,0.4,0.8 mmol/L) were applied to HepG2 cells for 24, 48 and 72 h to study its effect on cell viability using the MTT assay. Group of isoquercitrin with concentration of 0.2mmol/L could inhibit the proliferation of the cells, and its rate was 72.39%. After 48 h of treatment, group of isoquercitrin with concentration of 0.1 mmol/L had a significant inhibitory effect on cell proliferation, while the proliferation inhibitory rates of 0.2 and 0.4 mmol/L groups were 66.71% and 52.67% respectively. After 72 h of treatment, group of isoquercitrin with concentration of 0.05 nmol/L also significantly inhibited cell proliferation (Fig 1). This study showed that isoquercitrin decreased the capability of HepG2 cells in a time- and dose-dependent manner.

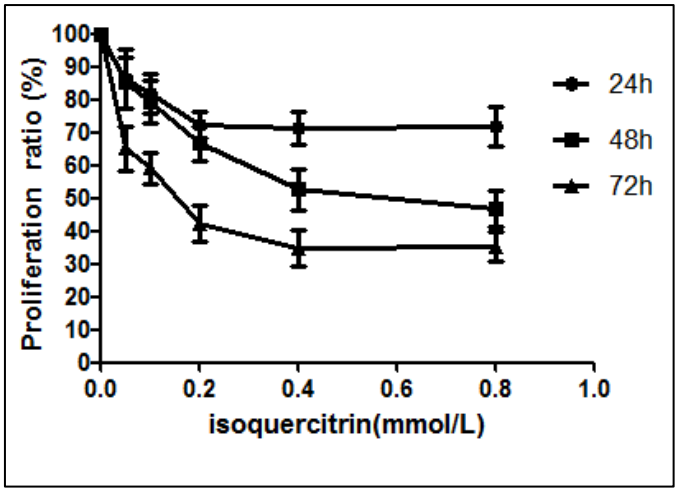


**Figure 1: The growth effect of HepG2 cells treated with Isoquercitrin. Different concentrations of isoquercitrin (0, 0.05, 0.1, 0.2, 0.4, 0.8 mmol/L) were used to treat HepG2 for 24, 48, and 72 h**

The therapeutic effects of isoquercitrin for cells on the expression of GRP78, PERK, elF2a, ATF4 and CHOP mRNA

In order to explore what the molecular mechanism of isoquercitrin causes cell apoptosis, the isoquercitrin with different concentrations to treat HepG2 cells for 48 hours. Fluorescence quantitative PCR was used to detect the expression of GRP78, PERK, elF2a, ATF4 and CHOP mRNA. The results showed that, compared with the blank group, the expression of GRP78, PERK, elF2a, ATF4 and CHOP mRNA increased gradually with the increase of isoquercitrin concentration. The results showed that isoquercitrin had the same effect on the expression of GRP78, PERK, elF2a, ATF4 and CHOP mRNA.

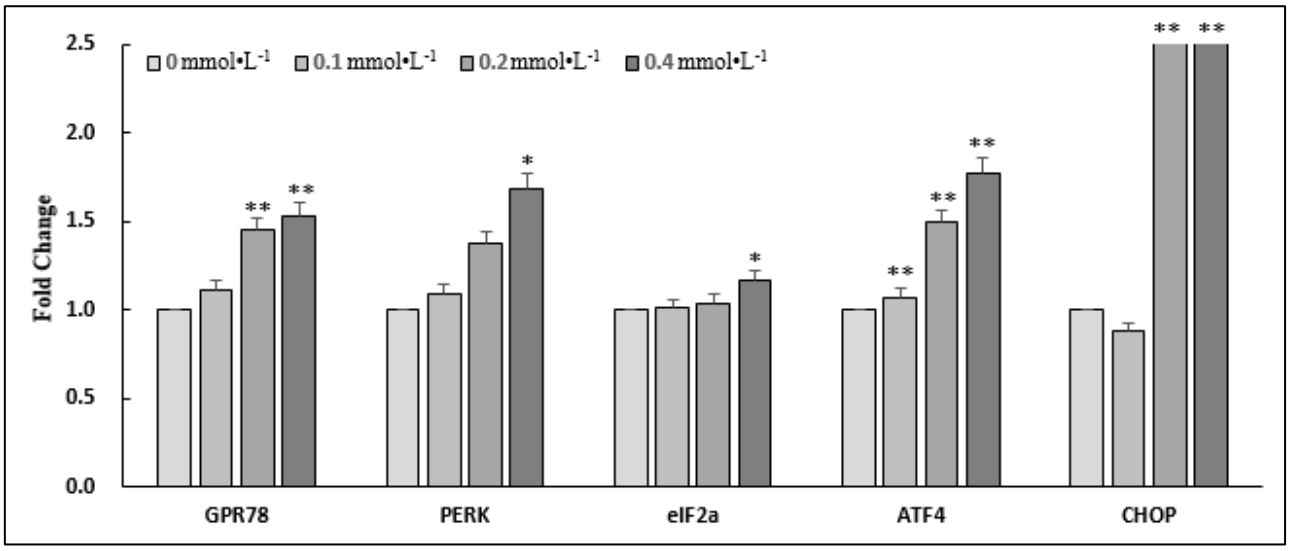


**Figure 2: Isoquercitrin inhibits HepG2 cells proliferation on the mRNA expression levels of GRP78, PERK, elF2a, ATF4 and CHOP. HepG2 cells were treated with different concentrations of isoquercitrin (0, 0.1, 0.2, 0.4 mmol/L) for 48 h. Quantitative PCR assay of GRP78, PERK, elF2a, ATF4 and CHOP mRNA expression levels. Compared with the control group, *p < 0.05 and **p < 0.01**

The therapeutic effects of isoquercitrin for cells on the expression of GRP78, PERK, elF2a, ATF4 and CHOP proteins

In order to explore what the molecular mechanism of isoquercitrin causes cell apoptosis, western blotting was used to detect the expression of GRP78, PERK, p-PERK, elF2a, ATF4 and CHOP proteins when cells were treated with different concentrations of isoquercitrin for 48 hours. The results showed that, compared with the blank group, with the increase of isoquercitrin concentration, the expression of GRP78, p-PERK, elF2a, ATF4, CHOP proteins was gradually increased, and the expression of PERK protein was decreased (Fig 3). The results showed that isoquercitrin induced cell apoptosis may be related to the above factors.

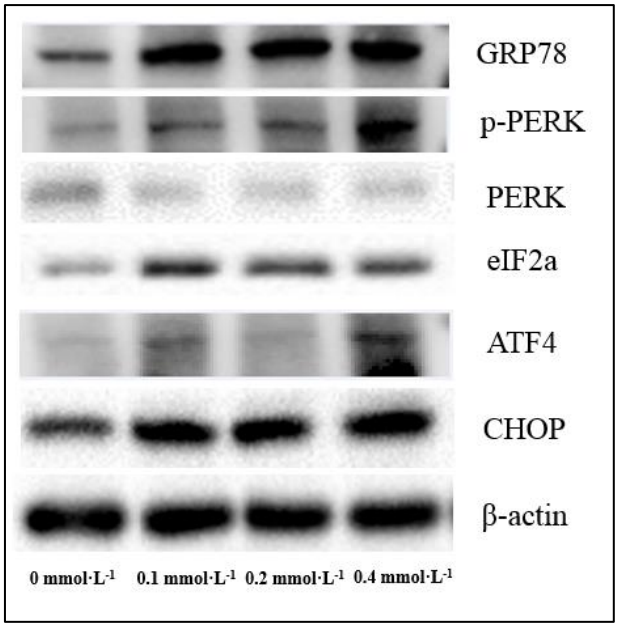


**Figure 3: Isoquercitrin inhibits HepG2 cells proliferation on the protein expression levels of GRP78, PERK, p-PERK, eIF2a, ATF4 and CHOP. HepG2 cells were treated with different concentrations of isoquercitrin (0, 0.1, 0.2, 0.4 mmol/L) for 48 h**

## DISCUSSION

In recent decades, a great deal of biology has been studying flavonoids, and their beneficial effects on human health are mainly attributed to their antioxidant and anti-inflammatory activities [2, 5]. Isoquercitrin (quercetin -3-O-b-D- glucopyranoside) can be obtained in many fruits, vegetables, Chinese herbal medicines and foods, and is a member of flavonoids. Studies have shown that isoquercitrin has the ability to scavenge active oxygen/nitrogen, including superoxide anion radical, hydroxyl radical, hydrogen peroxide radical and peroxynitrite radical [6-8]. In addition, some studies suggest that the antioxidant effect of flavonoids may play an active role in various diseases related to oxidative stress, such as inflammation, atherosclerosis and cancer [2]. Studies have shown that isoquercitrin has certain inhibitory effects on pancreatic cancer, bladder cancer, ovarian cancer and various precancers [9-11]. The results studied the function of isoquercitrin in inhibiting the growth of cancer cells, and found that isoquercitrin could inhibit the growth of cancer cells and the upstream factors of endoplasmic reticulum stress pathway.

Endoplasmic reticulum (ER) is intricately involved in the regulation of protein folding, protein translocation and calcium (Ca2+) homeostasis, and its main sign is the folding protein with accumulated errors. In order to avoid the growth of unfolded proteins in ER and stabilize cells, cells will trigger unfolded protein reaction (UPR) [12]. UPR includes three parallel steps: inhibiting protein synthesis to prevent the production of extra unfolded protein, promoting the refolding of unfolded protein by stimulating ER, and activating the ubiquitin-proteasome protein degradation pathway to remove the accumulated unfolded protein. The failure of UPR can trigger ER stress-induced apoptosis [13].

The main regulator of endoplasmic reticulum (ERS) response mechanism is the protein kinase RNA-like endoplasmic reticulum kinase initiation factor 2a (eIF2a) in eukaryotic cells, which activates transcription factor 4(ATF4) and C/EBP homologous protein (CHOP) [14]. In the process of endoplasmic reticulum stress, through phosphorylation and activation, PERK phosphorylates eIF2a at serine 51 (S51), which leads to the inhibition of cell initiation mechanism and the reduction of overall protein synthesis. Furthermore, the enhanced phosphorylation of eIF2a increased the translation of ATF4 mRNA and induced autophagy, oxidative stress and apoptosis [15].

The results showed that isoquercitrin could up-regulate the expression of GRP78, p-PERK, elF2a, ATF4, CHOP proteins and down-regulate the expression of PERK in a concentration dependent manner, indicating that the molecular mechanism of isoquercitrin induced apoptosis of HepG2 cells might be related to up-regulate the expression of GRP78, p-PERK, elF2a, ATF4, CHOP proteins and down-regulate the expression of PERK.

## CONCLUSION

The research showed that isoquercitrin could inhibit the proliferation and induce apoptosis of HepG2 cells, which may be related to the up-regulate the expression of GRP78, p-PERK, elF2a, ATF4, CHOP proteins and down-regulate the expression of PERK. This study provides a theoretical basis of the application of isoquercitrin in the treatment of liver cancer.

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