2015년 2월

석사학위논문

The cytoprotective effect of isosalipurposide via Nrf2 activation

조선대학교 대학원

약 학 과

한 재 윤

Nrf2 활성화를 통한 Isosalipurposide의 세포보호 효능

The cytoprotective effect of isosalipurposide via Nrf2 activation

2015년 2월 25일

조선대학교 대학원

약 학 과

한 재 윤

The cytoprotective effect of isosalipurposide via Nrf2 activation

지도교수 기 성 환

이 논문을 약학 석사학위신청 논문으로 제출함

2014년 10월

조선대학교 대학원

약 학 과

한 재 윤

한재윤의 석사학위논문을 인준함

위원장 조선대학교 교 수 최 홍 석 (인)

위 원 조선대학교 교 수 기 성 환 (인)

위 원 조선대학교 교 수 신 상 미 (인)

2014년 11월

조선대학교 대학원

Contents

List of Figures

List of Abbreviations

ARE	**Antioxidant response element**
GCL	**Glutamate cysteine ligase**
GSH	**Glutathione**
HO-1	**Hemeoxygenase-1**
HPLC	**High-performance liquid chromatography**
ISPS	**Isosalipurposide**
Nrf2	**NF-E2-related factor 2**
NQO1	**NAD(P)H:quinone reductase**
ROS	**Reactive oxygen species**
t-BHP	**Tert-butylhydroperoxide**

국문초록

Nrf2 활성화를 통한 Isosalipurposide의 세포보호 효능

한 재 윤

지도교수 : 기 성 환

약 학 과

조선대학교 대학원

본 연구실에서는 Chalcone 유도체인 isosalipurposide (ISPS)를 우리나라 자생식물인 히어리 (*Corylopsis coreana Uyeki)*로부터 성공적으로 분리하였다. ISPS의 항균성과 항증식성 효능에 대한 연구가 수행된 바가 있지만 구체적인 약리기능에 대한 연구가 전무한 실정이다. 본 연구에서는 ISPS의 NF-E2-related factor (Nrf2)-antioxidant response element (ARE) 신호 활성화 및 Nrf2 타겟 유전자들의 유도 여부를 관찰하였다. 또한 산화적 손상에 대한 ISPS의 보호효능을 간세포주를 활용하여 연구하였다. HepG2 세포에서 ISPS 처리는 Nrf2의 핵내 이동을 증가시켰다. 또한 ISPS는 ARE reporter gene의 활성과 Nrf2의 표적유전자인 glutamate cysteine ligase (GCL) 및 hemeoxygenase-1 (HO-1)의 발현량을 증가시켰다. GCL의 증가는 실제로 세포 내의 glutathione 양을

증가시켰다. ISPS의 전처리는 t-BHP에 의한 ROS 생성 및 glutathione의 감소를 유의적으로 회복시켰으며 ROS에 의한 미토콘드리아의 기능장애와 세포사멸을 회복시켰다. ISPS에 의한 Nrf2 활성화 및 세포보호 효능은 $Nrf2^{-/-}$ MEF 세포와 ARE 결손 reporter plasmid를 사용하여 증명하였다. 마지막으로 ISPS에 의한 Nrf2의 활성화에 ERK와 AMPK의 인산화가 요구됨을 확인할 수 있었다. 이 결과를 통해 본 연구에서는 간세포에서 ISPS가 Nrf2의 활성화 및 그 타겟 유전자의 발현을 통해 산화적인 손상에 대한 세포보호 효능이 있음을 입증함으로 ISPS가 다양한 간질환의 치료후보약물이 될 수 있음을 제시한다.

Ⅰ. Introduction

Balance of generation and elimination of reactive oxygen species (ROS) are important in maintaining normal cellular homeostasis. However, excessive ROS production and oxidative stress is associated with a variety of cellular activity including inflammatory responses and apoptosis which contributes to the many pathological conditions and diseases including aging, cancer and metabolic diseases. Antioxidant defense system protects the cells from oxidative stress mediated substantial damage (Trachootham et al., 2009; Birben E et al., 2012).

NF-E2-related factor (Nrf2) is a basic leucine zipper transcription factor, is encoded by the NFE2L2 gene in humans. Nrf2 is a master regulator of the antioxidant response due to fact that it regulates expression of several detoxification or antioxidant enzymes (Gorrini et al., 2013). Under normal or unstressed conditions, Nrf2 is negatively regulated through ubiquitination and degradation by Kelch-like ECH associated protein 1 (Keap1) ubiquitin E3 ligase complex. However, under oxidative stress, Nrf2 is released from Keap1 and induces translocation of Nrf2 into nucleus where it binds strongly to antioxidant response elements (ARE) sequence and regulates ARE-mediated antioxidant proteins and phase II detoxifying enzymes (Finkel, 2011; Valko et al., 2007; Jaramillo and Zhang, 2013).

Chalcones, one of the subclasses of flavonoid family, are regarded as the precursor of the flavones in the biosynthesis of flavonoids. These phenolic compounds all contain a 1,3-diphenyl-2-en-1-one framework and forms the central core for several important biological compounds obtained from plants (Yadav et al.,

2011). The chalcone derivatives have been reported to exhibit a broad spectrum of pharmacological properties and potential applications such as anti-microbial, anti-inflammatory, anti-platelet and antioxidant (Yadav et al., 2011; Singh et al., 2014). They have also been reported for the inhibition of several important enzymes including epoxide hydrolase, xanthine oxidase etc. It has been reported that electrophilic α, β-unsaturated carbonyl moiety on chalcone resulted in the activation of Nrf2 and the induction of phase II detoxifying enzyme expression (Kumar et al., 2011). In the current study, we successfully isolated isosalipurposide from *Corylopsis coreana* Uyeki (Korean winter hazel). Although isosalipurposide is known about the effect of anti-oxidative (Agnihotri et al., 2008) and anti-proliferative activity (Zhang et al., 2013), little is known about the pharmacolgical effect of isosalipurposide.

In this study, we investigated whether isosalipurposide protects against oxidative stress through Nrf2 activation and its target gene expression. In addition, to verify the role of Nrf2 activation by isosalipurposide, we used an ARE deletion mutant plasmid construct and Nrf2 knockout MEF cells. Nrf2 activation by isosalipurposide was found to protect against oxidative injury and to have a cytoprotective effect in hepatocytes. Furthermore, Nrf2 activation by isosalipurposide was found to be due to the phosphorylations of ERK, PKCδ, and AMPK. Collectively, our results demonstrate that isosalipurposide protects hepatocytes against oxidative stress by activating Nrf2 and inducing phase II antioxidant gene expression.

Ⅱ. Material & Methods

1. Materials

Rhodaimne (Rh123) and Antibodies against Nrf2, phospho-Nrf2 and PARP were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK 1/2, phospho-p38, lamin A/C and caspase-3 antibodies were obtained from Cell Signaling (Danvers,MA). Sestrin2 antibody was obtained from Proteinteck (Chicago, IL) and GCL antibody from Abcam (Cambridge,MA). HO-1 antibody was provided by Enzo Life Sciences (PlymouthMeeting, PA) and MTT, Rotenone, metaphosphoric acid, DCFH-DA, t-butylhydroquinone (t-BHQ), t-butylhydroperoxide (t-BHP), dimethylsulfoxide and β-actin antibody were purchased from Sigma Chemicals (St. Louis, MO).

2. Preparation of isosalipurposide

The air-dried, powdered *Corylopsis coreana Uyeki* flos (200 g) were extracted twice with 70% ethanol by sonication for 3 hours. After filtration, the extract was evaporated and suspended in distilled water and then defatted with *n*-hexane. The aqueous layer was partitioned with ethylacetate. The evaporation residue (5 g) was subjected to flash silica gel chromatography, using *n*-hexane: chloroform: MeOH solvent system (2:1:0.1~1:1:0.1~100% MeOH). Isosalipurposide was further purified by preparative thin layer chromatography to an analytically acceptable purity. Identification and purification of the isosalipurposide was conducted by thin layer chromatography using hexane, chlroroform and methanol as a solvent system. Spots were detected by UV at 254 and 366nm. HPLC system was used for the

analysis of isosalipurposide. The liquid chromatography used throughout consisted of a Waters separations module, automatic sample injector and a UV detector with peak integration at 350 nm for quantitative analysis. Data were analyzed with an integrator (Waters corporation).A zorbax extended RP-18 column (150 x 4.6mm, 5 μm, Agilent) was used for separation. The mobile phase was methanol (solution A) and water containing 0.1% acetic acid (solution B). The flow rate was 1.0 mL/min. and the injection volume was 10 μL. The gradient elution condition is summarized in Table 1. After completing the chromatographic elution, the mobile phase was programmed to its initial condition within 5 min. Isosalipurposide was subjected to spectroscopic analysis: 300 MHz ^{1}H NMR and 75 MHz ^{13}C NMR were determined at Korea Basic Science Institute, Korea.

3. Cell Culture

HepG2 cell lines were purchased from the ATCC (the American Type Culture Collection, Manassas, VA). Nrf2 knockout and wild-type (WT) MEF cells were kindly donated by Dr. MK Kwak (Catholic University, South Korea). Cells were plated at 1 × 10^5 per well in six-well plates, and used when 70%–80% confluent. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. ISPS, dissolved in dimethylsulfoxide (DMSO), was added to cells and incubated at 37 °C for the indicated time period. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) before sample preparation.

4. Immunoblot Analysis

Cell lysates and subcellular fractions were prepared according to the previously published methods (Ki et al., 2005; Shin et al., 2012). SDS–polyacrylamide gel electrophoresis and immunoblot analyses were performed according to previously published procedures (Shin et al., 2012). Briefly, the cell lysates were separated by 7.5% and 12% gel electrophoresis and blots were electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with primary antibody as indicated, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA). Immunoreactive protein was visualized by an ECL chemiluminescence detection kit (AmershamBiosciences, Buckinghamshire, UK). Equal loading of proteins was verified by Coomassie blue staining of gels and β-actin or lamin A/C immunoblotting. At least three separate experiments were performed with different lysates or subcellular fractions to confirm changes in the protein levels.

5. Plasmid Construction and Luciferase Assay

The hSesn2 promoter-driven luciferase construct (pGL4-phSesn2) and deletion mutants of Sesn2 promoter-luciferase plasmid (pGL3-phSesn2-ΔARE), in which ARE were deleted, were constructed by PCR-based methods as previously reported (Shin et al., 2012). To determine the luciferase activities, we used the dual-luciferase reporter assay system (Promega). Briefly, HepG2 cells were re-plated in 12-well plates overnight, serum-starved for 6 h, and transiently transfected with each Sesn2 promoter-luciferase construct and pRL-SV plasmid (a plasmid that encodes for Renilla luciferase and is used to normalize transfection efficacy) in the

presence of Lipofectamine® Reagent (Invitrogen, San Diego, CA) for 3 h. NQO1-ARE luciferase construct, 3-tandem repeat of ARE in the 5'-upstream region of NQO1, was introduced into the cells to examine transcriptional activation of Nrf2 by isosalipurposide. Transfected cells were incubated in DMEM containing 1% fetal bovine serum (FBS)(Hyclone, Logan, UT) for 3 h and exposed to ISPS for 12 h. Firefly and Renilla luciferase activities in cell lysates were measured using a luminometer (Promega, Madison, WI). The activity of firefly luciferase was measured by adding Luciferase Assay Reagent II (Promega) according to the manufacturer's instructions, and the Renilla luciferase reaction was initiated by adding Stop & Glo® reagent (Promega, Madison, WI). Relative luciferase activities were calculated by normalizing firefly luciferase activity with that of Renilla luciferase.

6. MTT assay

To measure cytotoxicity, HepG2 cells or MEF cells were plated at a density of 1 × 10^5 cells/well in 48-well plates and treated with t-BHP (500 μM, 12 h) in the presence or absence of isosalipurposide (10-100 μM, 1 h pretreatment). After treatment, viable cells were stained with MTT (0.2 mg/ml, 4 h). The media were then removed, and formazan crystals produced in the wells were dissolved with the addition of 200 μl of dimethyl sulfoxide. Absorbance at 540 nm was measured using an enzyme-linked immunosorbent assay microplate reader (SpectraMAX, Molecular Device, Sunnyvale, CA). Cell viability was defined relative to untreated control [i.e., viability (% control) = 100 × (absorbance of treated sample) / (absorbance of control)].

7. Determination of GSH content

The GSH contents in the cells were quantified using a commercial GSH determination kit (BIOXYTECH GSH-400, Oxis International). Cells were plated onto 6-well dishes (1×10^6 cells/well) and were exposed to 500 μM t-BHP for 12 h following 1 h of treatment with 10-100 μM ISPS or ISPS alone. Scraped cells were lysed in buffer containing 5% metaphosphoric acid to precipitate proteins. After being centrifuged at 10,000 × g for 10 min, the supernatants were used to measure GSH concentration. Absorbance at 400 nm was measured using a microplate reader (SpectraMAX, Molecular Device, Sunnyvale, CA).

8. Measurement of ROS production

Cells grown on 12-well plates were treated with 10 μM DCFH-DA for the last 1 h of each treatment at 37 °C and were harvested by trypsinization. The collected cells were washed twice with wash buffer and H_2O_2 generation was determined by using a fluorescence microplate reader (Jemini, Molecular Device) with excitation/emission wavelengths of 485/530 nm. ROS production was normalized with protein concentration of each treated sample and defined as relative to vehicle-treated control

9. Analysis of mitochondrial membrane permeability change

Changes in mitochondrial membrane permeability were measured with Rh123, a membrane-permeable cationic fluorescent dye. Cells were treated according to the individual experiment, and were stained with 0.05 μg/ml Rh123 for 1 h. Cells were

harvested by trypsinization and washed twice with wash buffer. The changes in mitochondrial membrane permeability were measured using a fluorescence microplate reader (Gemini XPS, Molecular Devices, Sunnyvale, CA). The fluorescence intensity of Rh123 was calculated as described in ROS production.

10. Statistical Analysis

One way analysis of variance (ANOVA) was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data were expressed as means ± S.D.

Ⅲ. Results

1. Nrf2 activation and target gene induction by isosalipurposide.

To examine the effect of ISPS on Nrf2 activation, we first treated HepG2 cells with several concentration of ISPS for 3 h and then examined the dose-response effect of ISPS on the nuclear accumulation of Nrf2. Treatment with ISPS increased nuclear Nrf2 levels in a dose-dependent manner (Fig 2A). Next, we treated HepG2 cells with 100 μM of ISPS for 0-6 h and then examined the effect of ISPS on the nuclear accumulation of Nrf2 (Fig. 2A). It was found that nuclear Nrf2 levels were increased by ISPS treatment. Then, we performed reporter gene analysis for ARE to verify ISPS-induced NQO1-ARE activation. NQO1-ARE luciferase constructs that contain 3-tandem repeats of ARE in the 5′-upstream region of NQO1 were transfected into HepG2 cells to examine transactivation by ISPS. Exposure of transfected cells to ISPS resulted in a significant increase in luciferase activity of the NQO1-ARE reporter construct (Fig 2B). Next, we found that Nrf2 target gene expression including GCL and HO-1 was induced by ISPS treatment (Fig 2C). GCL is the rate-limiting enzyme for glutathione biosynthesis and its expression is mainly regulated by the Nrf2-ARE pathway (Wild et al., 1998). We measured intracellular GSH levels and results indicated that ISPS directly increased GSH level in HepG2 cells (Fig 2D).

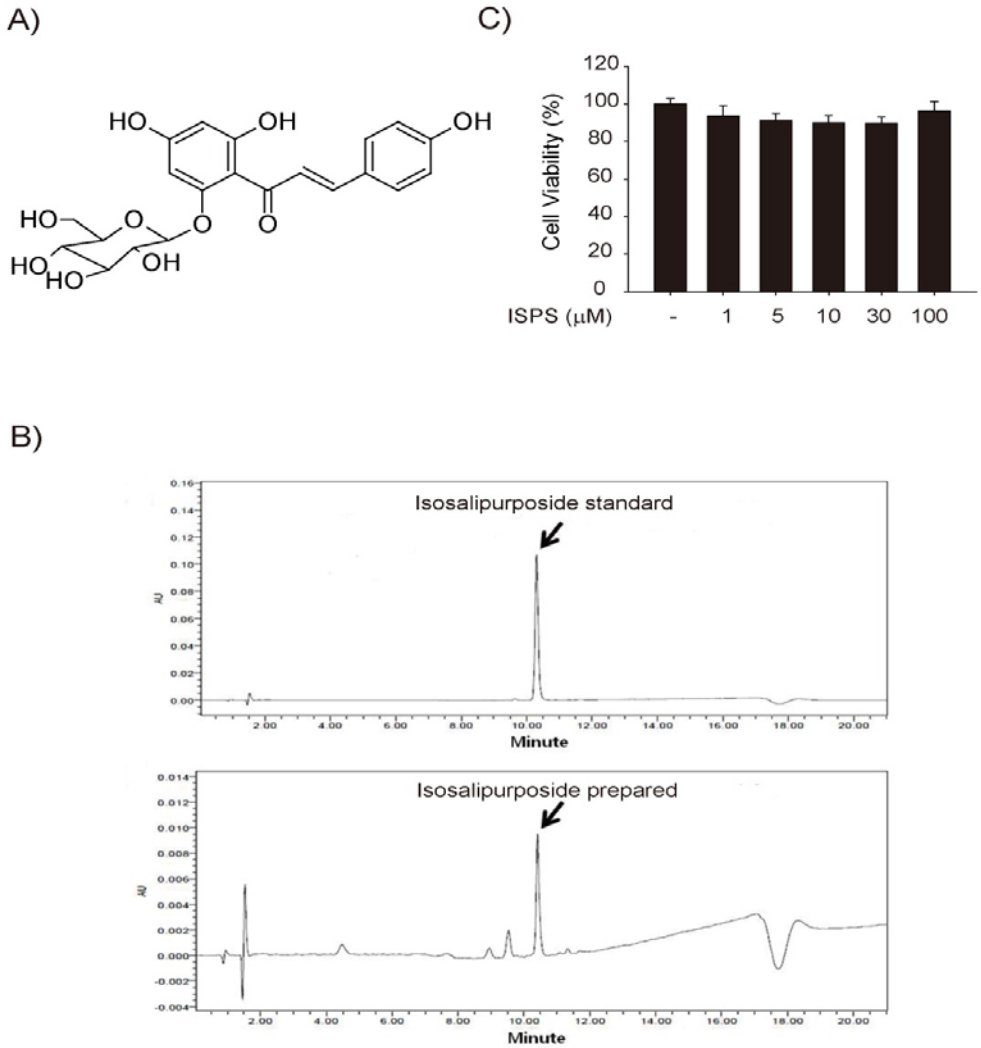


Figure 1. The effect of isosalipurposide (ISPS) on cytotoxicity

(A) Chemical structure of ISPS. (B) Identification of ISPS by HPLC. (C) MTT assays for cell viability. The effect of ISPS (10~100 μM, 24 hr treatment) on cell viability was assessed using MTT assays

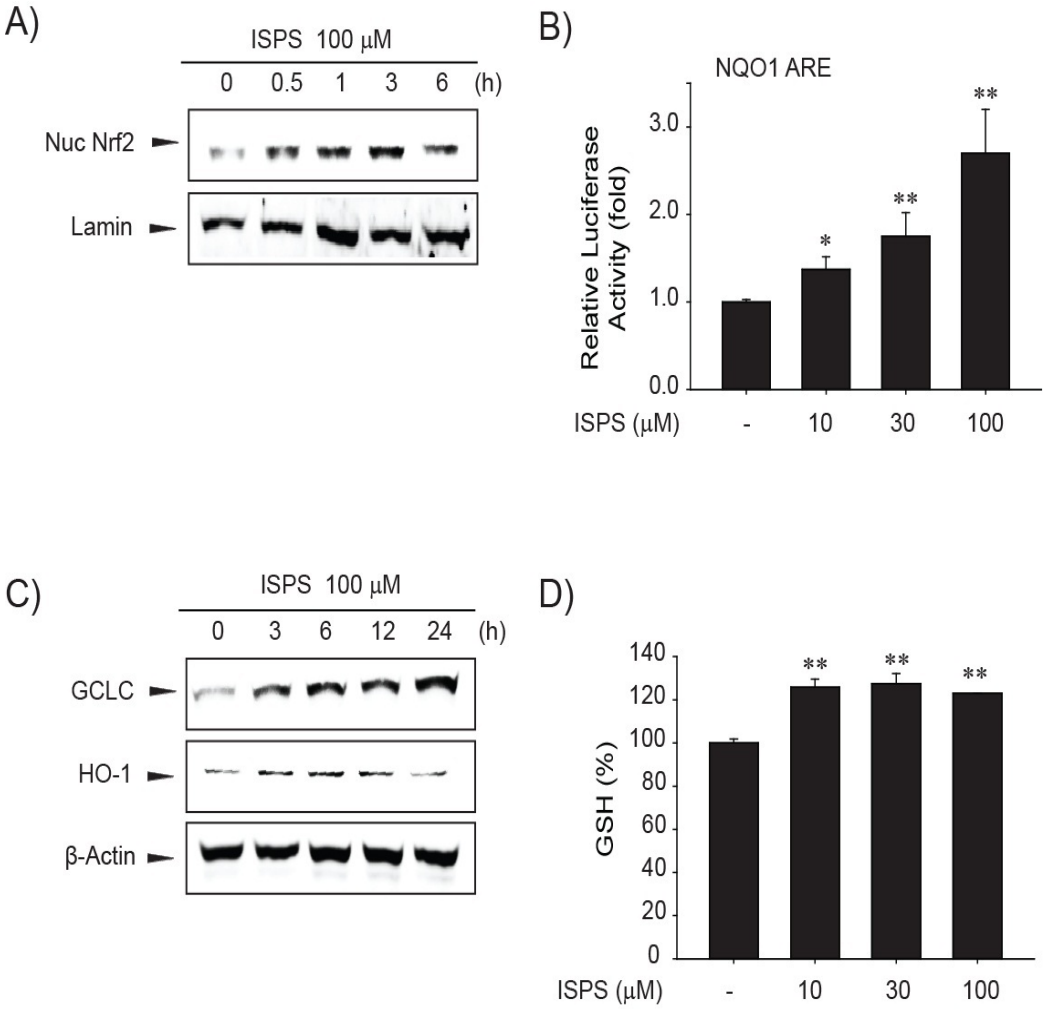


Figure 2. The effect of isosalipurposide (ISPS) on Nrf2 activation and target gene expression.

(A) The effect of varying times of ISPS on the nuclear Nrf2 translocation in HepG2 cells. Nrf2 protein was immunoblotted in the nuclear fractions of cells incubated in the presence of 100 μM of ISPS for 0 min to 6 h. (B) Increases in Nrf2 transactivation by ISPS. NQO1-ARE luciferase activity were measured on the lysates of HepG2 cells transfected with the NQO1-ARE luciferase construct exposed to 10-100 μM ISPS for 12h. (C) The effect of ISPS on the expression of Nrf2 target genes. HepG2 cells were treated with 100 μM of isosalipurposide for 0 min to 24 h. GCLC and HO-1 were immunoblotted from the lysates of cells. (D) Increases in the intracellular GSH level by ISPS. GSH concentrations were measured in the lysates of cells treated with 10–100 μM of ISPS for 12 h. Data represent the mean ± SD of four replicates; the statistical significance of differences between each treatment group and the control (**P < 0.01).

2. Inhibition of oxidative stress and mitochondrial dysfunction by isosalipurposide

ROS-mediated oxidative damage is a mechanism to cause cellular damage and mitochondrial dysfunctions (Cervinoka et al., 2007). *tert*-Butyl hydroperoxide induces oxidative stress, which may cause a significant increase in the ROS formation (Davies, MJ., 1989). Therefore, we measured whether ISPS restored ROS production and GSH concentration in cells treated with t-BHP. Induced ROS production (Fig 3A) and depleted intracellular GSH level (Fig 3B) by t-BHP was prevented by ISSP pretreatment. Next, we investigated changes in mitochondrial membrane permeability transition using Rhodamine 123 (Rh123). Rh123 is cell-membrane permeable and localizes in mitochondria of cells to emit yellowish-green fluorescence (Kwon et al, 2009, Lemasters and Nieminen, 1997). Since low intensity of Rh123 indicates mitochondrial damage, we used Rh123 as a probe of mitochondrial membrane potential of cells. Intensity of Rh123 fluorescence was decreased by t-BHP, but ISPS restored decreased Rh123 fluorescence, which represents that ISPS inhibited the mitochondrial permeability transition induced by t-BHP (Fig 3C). Rotenone is an inhibitor of complex 1 in the mitochondrial respiratory chain. To find evidence that the protective effect of ISPS on mitochondrial dysfunction, we adopted rotenone. Treatment with rotenone induced cell death, which was reversed by ISPS (Fig 3D). These results show that t-BHP-induced ROS production and cell death through mitochondrial dysfunction was antagonized by ISPS.

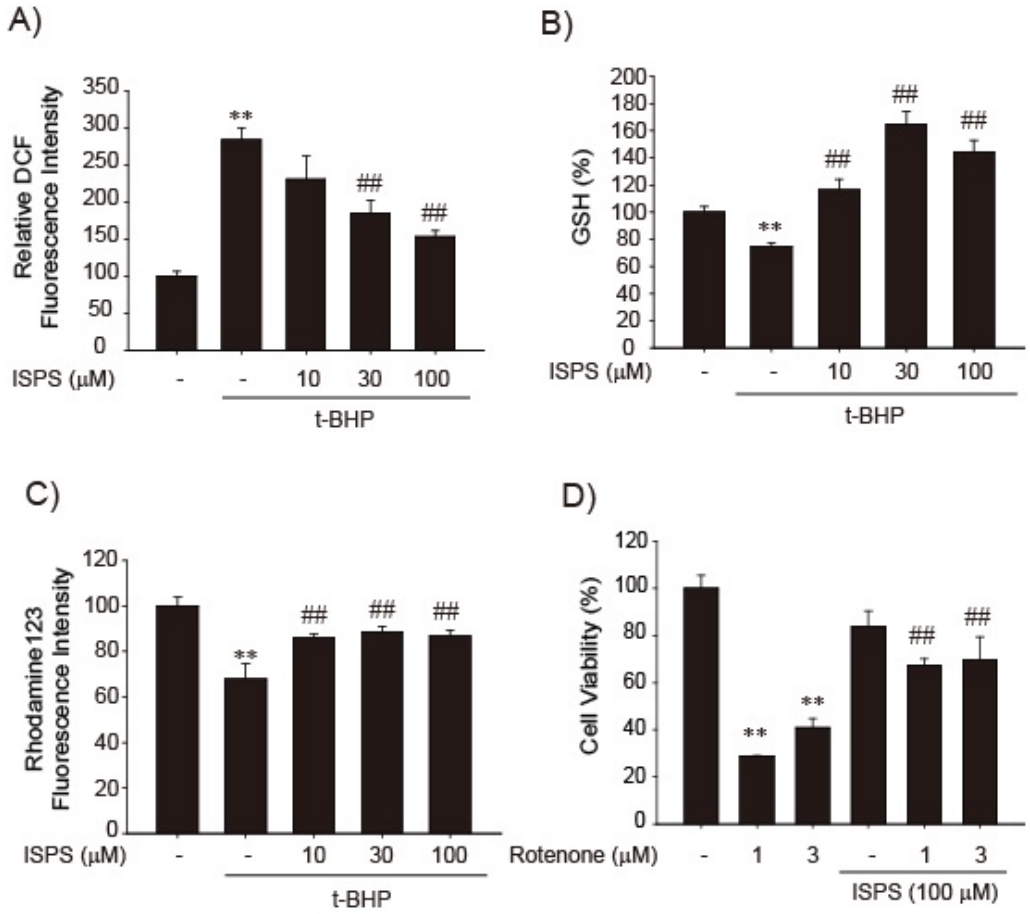
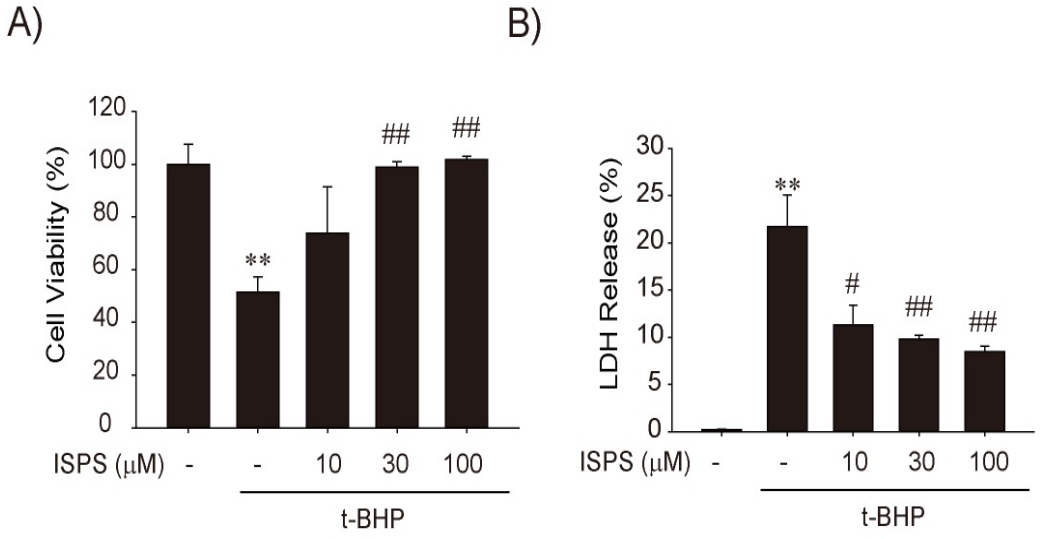


Figure 3. The inhibitory effect of isosalipurposide (ISPS) on ROS production.

(A) The effect of ISPS on t-BHP-induced ROS production. HepG2 cells were treated with 500 μM t-BHP and/or 10–100 μM ISPS. Cells were stained with 10 μM DCFH-DA for 30 min at 37 °C. (B) The GSH level was measured in lysates of cells treated with 500 μM t-BHP and/or 10–100 μM ISPS for 12 h. (C) Measurement of mitochondrial membrane permeability changes. HepG2 cell were treated with 500 μM t-BHP and/or 10–100 μM ISPS for 12 h. After staining with Rhodamine123, the cells were harvested and then changes in mitochondrial membrane permeability were determined by fluorescence reader. (D) MTT cell viability. Cells were treated with 1-3 μM rotenone and/or 100 μM ISPS for 24 h. The effect of ISPS on rotenone-induced cell death was assessed using MTT assays. Data represent the mean ± SD of four replicates; **P<0.01, significant versus vehicle-treated control; ##P<0.01 significant versus t-BHP or Rotenone alone.

3. Inhibition of t-BHP-induced cell death by isosalipurposide.

To study the protective effects of ISPS on t-BHP-induced cell death, cells were pretreated with various concentrations of isosalipurposide (10-100 μM) for 1 h, followed by incubation with 500 μM of t-BHP for 12 h. Cell viability and cell membrane permeability were examined by colorimetric MTT and LDH release, respectively. ISPS prevented t-BHP-induced cell death (Fig 4A). Cells treated with t-BHP resulted in significant increases in LDH release, while pretreatment with 10-100 μM ISPS significantly inhibited t-BHP-induced LDH release. Moreover, we investigated ISPS inhibition of t-BHP-induced oxidative damage was associated with the induction of apoptosis, cell lysates were immunoblotted for apoptosis marker proteins, such as PARP and caspase-3. Compared to the control, t-BHP exposure decreased expression of PARP and caspase-3. However, ISPS treatment prevented these events by t-BHP (Fig 4C). Consistently, ISPS inhibited the activation of caspase 3 by t-BHP treatment.



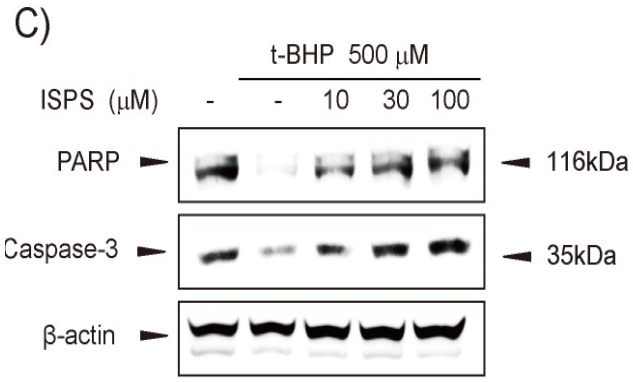


Figure 4. The cytoprotective effect of isosalipurposide (ISPS).

(A) MTT cell viability. The effect of ISPS (10–100 μM, 1 h pretreatment) in the presence or absence of t-BHP (500 μM, 6 h) on cell viability was assessed using MTT assays. (B) LDH assay. Cell membrane permeability was assessed by determining the amount of LDH. HepG2 cells were incubated with 500 μM t-BHP and/or 10–100 μM ISPS. Data represent the mean ± SD of four replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus t-BHP alone. (C) Immunoblots of apoptotic proteins. Precursor PARP and procaspase-3 were immunoblotted in the lysates of cells incubated with 10–100 μM ISPS for 1 h, and then treated with 500 μM t-BHP for 6 h. Equal protein loadings were confirmed by immunoblotting for β-actin.

4. Role of Nrf2 on cytoprotective effect by isosalipurposide.

To explore the role of Nrf2 on cytoprotective effect by ISPS, we adopted Nrf2 knockout MEF cells. Nrf2 deficiency blocked ISPS-induced HO-1 expression (Fig 5A). Next, we examined cytoprotective effect of ISPS in Nrf2 knockout MEF cells. Cells treats with ISPS 100 μM and/or t-BHP 500 μM. MTT assays show ISPS restored decreased cell viability by oxidative stress in wild-type MEF cells. However, Nrf2 deficiency failed to recover (Fig 5B). These results indicate that the cytoprotective effect of ISPS required Nrf2 activation and target gene expression.

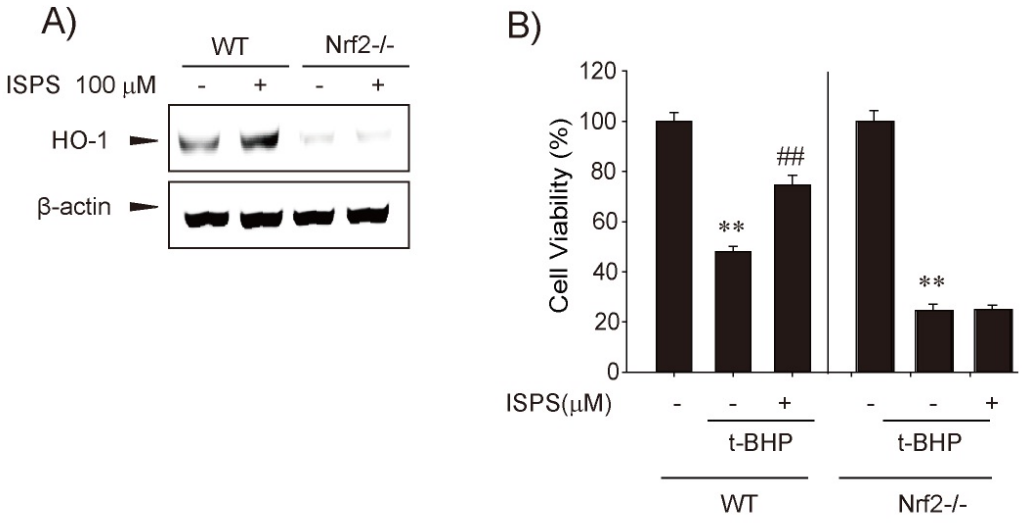


Figure 5. The role of Nrf2 on protective effect of isosalipurposide (ISPS).

(A) The effect of ISPS on HO-1 induction in wild type (WT) and Nrf2 knockout(Nrf2$^{-/-}$) MEF cells. HO-1 was immunoblotted with the lysates of WT or Nrf2$^{-/-}$ MEF cells treated with ISPS for 12 h. (B) The effect of ISPS (100 μM, 30 min pretreatment) in the presence or absence of t-BHP (500 μM, 6h) on cell viability was assessed using MTT assays in wild type (WT) and Nrf2 knockout MEF cells. Data represent the mean ± SD of four replicates; **P < 0.01, significant versus vehicle-treated control; ##P < 0.01, significant versus t-BHP alone.

5. Nrf2 phosphorylation by ERK1/2 and AMPK by isosalipurposide

The Nrf2 phosphorylation leads to dissociate of Nrf2 from Keap1 and thus, the nuclear translocation of Nrf2 and the activation of its transcriptional function (Niture et al., 2010). ISPS gradually increased Nrf2 phosphorylation from 30 min to 6 h (Fig 6A). To identify mechanism underlying Nrf2 phosphorylation by ISPS, we examined whether ISPS-induced phosphorylation of upstream kinase related to Nrf2 activation. ISPS increased phosphorylation of ERK and AMPK but not PKC and other MAPKs (Fig 6B).

.

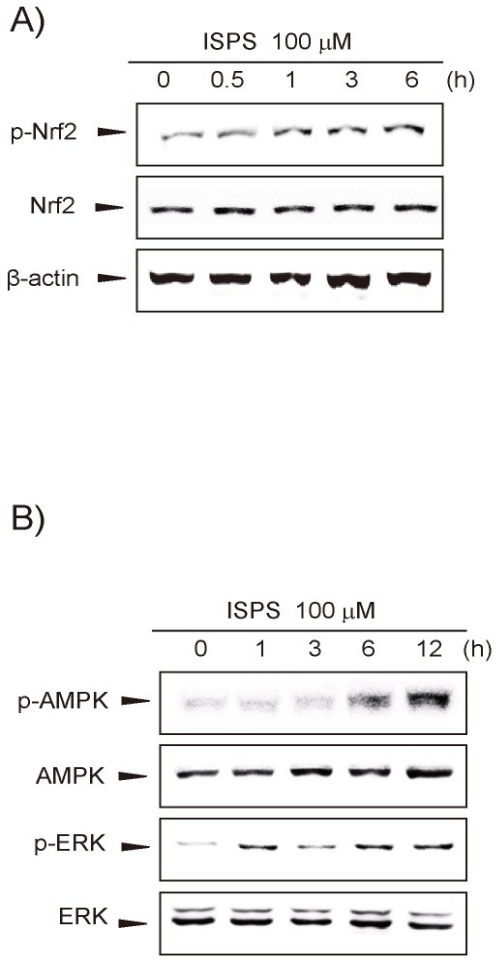


Figure 6. The activations MAPK and AMPK by isosalipurposide (ISPS).

(A) Immunoblot analysis for Nrf2 phosphorylation by ISPS. Cells were treatment with ISPS 100 μM for 30 min to 6 h. (B) The effect of ISPS on the phosphorylation of upstream kinases. Cells were treatments with 100 μM isosalipurposide for 1 to 12 h. The protein expression of total and phosphorylated MAPK (ERK and AMPK) was determined by western blotting.

Ⅳ. Discussion

Chalcone derivatives have been received much attention for its numerous positive effects in health including on inflammation and cancer, cardiovascular disease, diabetes, obesity, pulmonary disease, and neurologic disease.(Yamamoto et al., 1991; Yang et al., 2001; Herencia et al., 1999). In the present study, we isolated ISPS from *Corylopsis coreana* Uyeki (Korean winter hazel) which is native and endemic species of the Korean peninsula and is designated a Category Endangered Plant Species by the Wildlife Protection Act of South Korea. Although a broad range of biological activities of chalcones have been reported, little is known about the pharmacolgical effect of ISPS. Here, we investigated whether ISPS activates the redox sensitive transcription factor Nrf2, thereby protecting against oxidative damage in hepatocytes.

Although Nrf2 is well known multi-organ protector in response to oxidative stress, Nrf2 expression is especially enriched in metabolically active organs, such as the liver (Lee et al., 2005; Shin et al., 2013). In addition, it has been well established that oxidative stress plays an important role in the pathogenesis of a diversity of liver diseases. (Zhu et al., 2012). ISPS increased nuclear Nrf2 levels and NQO1-ARE luciferase activity. Activation of Nrf2 was associated with augmentation of its downstream targets, such as GCLC and HO-1. Overexpressed GCLC leads to increase intracellular GSH levels. The role of Nrf2 activation by ISPS was confirmed using Nrf2 KO MEF cells and ARE deletion mutant plasmid. Blockade of Nrf2 expression or ARE completely abolished ISPS-induced HO-1 expression or luciferase activity, respectively.

Diverse protein kinases have been implicated in the transduction of oxidative

stress signals to ARE-mediated gene expression. Moreover, a number of reports have addressed the possible roles played by extracellular signal-regulated kinase (ERK1/2) (Zipper and Mulcahy, 2003) protein kinase C delta (PKCδ) (Huang et al., 2002), phosphoinositide 3-kinase (PI3K) (Nakaso et al., 2003) and 5′ AMP-activated protein kinase (AMPK) (Liu et al., 2011) in Nrf2 activation. Here, we show that isosalipurposide increases the phosphorylation of Nrf2 which is accompanied by ERK and AMPK phosphorylation. Furthermore, ARE-mediated gene expression was inhibited by specific chemical inhibitors of ERK or AMPK, which confirmed the role played by these kinases in Nrf2 activation by ISPS.

Since ROS are mainly generated as by-products of mitochondrial respiration, mitochondria are considered to the primary target of oxidative injury and play an important role in the etiology of numerous diseases, such as cancer, metabolic syndrome, and aging (Seo et al., 2014; Murphy, 2009) In this study, we proved the ability of ISPS to improve t-BHP-induced mitochondrial dysfunction and related toxicity. Exposure of t-BHP induced ROS production and disrupted mitochondrial membrane potential, which were reversed by treatment with ISPS. Indeed, mitochondrial respiratory chain complex I inhibitor rotenone-induced cell death was significantly inhibited by ISPS.

Organic hydroperoxidant t-BHP is a well knowns ROS inducer, and it provokes oxidative stress in organ pathophysiology (Roy and Sil, 2012). Treatment with t-BHP mimics several aspects associated with liver pathology characterized by increased lipid peroxidation and cytotoxicity due to oxidative stress (Yamamoto and Farber, 1992; Rush et al., 1985). t-BHP decreased GSH and increased MDA level contribute to the loss of MMP which induce cell death via apoptosis (Kanupriya et al., 2007). The role of ISPS on cell viability and cell membrane permeability were analyzed using colorimetric MTT and LDH release, respectively. Treated with t-BHP

led to cell death, whereas the pretreatment with ISPS prevented them. In addition ISPS treatment, prevented t-BHP induced apoptosis markers, such as, PARP cleavage and cleaved form of caspase-3. These results support the notion that the cytoprotective effect of ISPS is mainly due to its antioxidative effects, although further studies are still needed to investigate the efficacy and effectiveness of ISPS using pathological animal models for liver diseases.

Collectively, the present study clearly shows that ISPS activates the Nrf2-ARE signaling pathway via the phosphorylation of ERK1/2 and AMPK in hepatocytes. Furthermore, ISPS inhibited t-BHP-induced mitochondrial dysfunction, ROS production and cell death. Our data strongly indicate that ISPS treatment may become a promising therapeutics to effectively prevent or treat oxidative stress-mediated liver diseases.

Ⅴ. Reference

1. Trachootham, D., Alexandre, J., Huang, P., 2009. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nature Reviews Drug Discovery 8, 579-591

2. Birben, E., Sahiner, UM., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organ J 5:9-19

3. Gorrini, C., Harris, IS., Mak, TW., Modulation of oxidative stress as an anticancer strategy. Nature Reviews Drug Discovery 12, 931-47

4. Finkel, T., 2011. Signal transduction by reactive oxygen species. J. Cell Biol. 194, 7-15.

5. Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84

6. Jaramillo, MC., Zhang, DD., 2013 The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev. 27:2179-91

7. Yadav, VR., Prasad, S., Sung, B., Aggarwal, BB., 2011. The role of chalcones in suppression of NF-κB-mediated inflammation and cancer. Int Immunopharmacol 11(3):295-309

8. Singh, P., Anand, A., Kumar, V., 2014. Recent developments in biological activities of chalcones: a mini review. Eur J Med Chem 85:758-77

9. Kumar, V., Kumar, S., Hassan, M., Wu, H., Thimmulappa, RK., Kumar, A., Sharma, SK., Parmar, VS., Biswal, S., Malhotra, SV., 2011. Novel chalcone derivatives as potent Nrf2 activators in mice and human lung epithelial cells. J Med Chem. 23;54(12):4147-59

10. Agnihotri, VK., Elsohly, HN., Khan, SI., Smillie, TJ., Khan, IA., Walker, LA., 2008. Antioxidant constituents of Nymphaea caerulea flowers. Phytochemistry. 69(10):2061-6

11. Zhang, EH., Wang, RF., Guo, SZ., Liu, B., 2013. An update on antitumor activity of naturally occurring chalcones. Evid Based Complement Alternat Med. 2013;2013:815621

12. Ki, S.H., Cho, I.J., Choi, D.W., Kim, S.G., 2005. Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. Mol. Cell. Biol. 25, 4150–4165.

13. Shin, B.Y., Jin, S.H., Cho, I.J., Ki, S.H., 2012. Nrf2-ARE pathway regulates induction of Sestrin-2 expression. Free Radic. Biol. Med. 53, 834–841.

14. Wild, A.C., Gipp, J.J., Mulcahy, T., 1998. Overlapping antioxidant response element and PMA response element sequences mediate basal and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase catalytic subunit gene. Biochem. J. 332 (Pt 2), 373–381.

15. Cervinkova, Z., Lotkova, H., Kriavkova, P., Rousar, T., Kucera, O., Tichy, L., Cervinka, M, Drahota, Z., 2007. Evaluation of mitochondrial function in isolated rat hepatocytes and mitochondria during oxidative stress. Altern Lab Anim. 35(3):353-61.

16. Davies, MJ., 1989. Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with rat liver microsomal fractions. Biochem J. 1989 Jan 15;257(2):603-6.

17. Kwon, Y.N., Shin, S.M., Cho, I.J., Kim, S.G., 2009. Oxidized metabolites of oltipraz exert cytoprotective effects against arachidonic acid through AMP-activated protein kinase-dependent cellular antioxidant effect and mitochondrial protection. Drug Metab. Dispos. 37, 1187–1197.

18. Lemasters, J.J., Nieminen, A.L., 1997. Mitochondrial oxygen radical formation during reductive and oxidative stress to intact hepatocytes. Biosci. Rep. 17, 281–291.

19. Niture, S.K., Kaspar, J.W., Shen, J., Jaiswal, A.K., 2010. Nrf2 signaling and cell survival. Toxicol. Appl. Pharmacol. 244, 37–42.

20. Yamamoto, S., Aizu, E., Jiang, H., Nakadate, T., Kiyoto, I., Wang, JC., Kato, R., 1991. The potent anti-tumor-promoting agent isoliquiritigenin. Carcinogenesis 12**:**317–323.

21. Yang, EB., Guo, YJ., Zang, K., Chen, YZ., Mack, P., 2001. Inhibition of epidermal growth factor receptor tyrosine kinase by chalcone derivatives. Biochim Biophys Acta 1150**:**144–152.

22. Herencia, F., Ferrándiz, ML., Ubeda, A., Guillén, I., Dominguez, JN., Charris, JE., Lobo, GM., Alcaraz, MJ., 1999. Novel anti-inflammatory chalcone derivatives inhibit the induction of nitric oxide synthase and cyclooxygenase-2 in mouse peritoneal macrophages. FEBS Lett 453:129-34.

23. Lee, J.M., Li, J., Johnson, D.A., Stein, T.D., Kraft, A.D., Calkins, M.J., Jakel, R.J., Johnson, J.A., 2005. Nrf2, a multi-organ protector? FASEB J. 19, 1061–1066.

24. Shin, SM., Yang, JH., Ki, SH., 2013. Role of the Nrf2-ARE Pathway in Liver Diseases. Oxid Med Cell Longev 2013:763257.

25. Zhu, R.,Wang, Y., Zhang, L., Guo, Q., 2012. Oxidative stress and liver disease. Hepatol. Res. 42, 741–749.

26. Zipper, L.M., Mulcahy, R.T., 2003. Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. Toxicol. Sci. 73, 124-134.

27. Huang, H.C., Nguyen, T., Pickett, C.B., 2000. Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. Proc. Natl. Acad. Sci U. S. A. 97, 12475-12480.

28. Nakaso, K., Yano, H., Fukuhara, Y., Takeshima, T., Wada-Isoe, K., Nakashima, K., 2003. PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. FEBS Lett. 546:181-4.

29. Liu X.M., Peyton, K.J., Shebib, A.R., Wang, H., Korthuis, R.J., Durante, W., 2011. Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. Am. J. Physiol. Heart Circ. Physiol. 300, H84-H93.

30. Seo, K., Ki, SH., Shin, SM., 2014. Methylglyoxal induces mitochondrial dysfunction and cell death in liver. Toxicol. Res. 30(3): 193-8.

31. Murphy M.P., 2009. How mitochondria produce reactive oxygen species.

Biochem J. 417:1-13.

32. Roy, A., Sil, PC., 2012. Tertiary butyl hydroperoxide induced oxidative damage in mice erythrocytes: Protection by taurine. Pathophysiology 19(2):137-48.

33. Yamamoto, K., Farber, JL., 1992. Metabolism of pyridine nucleotides in cultured rat hepatocytes intoxicated with tert-butyl hydroperoxide. Biochem Pharmacol. 43(5): 1119-26.

34. Rush, GF., Gorski, JR., Ripple, MG., Sowinski, J., Bugelski, P., Hewitt, WR., 1985. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. Toxicol Appl Pharmacol. 78(3):473-83.

35. Kanupriya., Prasad, D., Sai, Ram, M., Sawhney, R.C., Ilavazhagan, G., Banerjee, P.K., 2007. Mechanism of tert-butylhydroperoxide induced cytotoxicity in U-937 macrophages by alteration of mitochondrial function and generation of ROS. Toxicol In Vitro. 21(5):846-54.

ABSTRACT

The cytoprotective effect of isosalipurposide via Nrf2 activation

Han Jae Yun

Advisor : Prof. Ki Sung Hwan, Ph.D.

Department of Pharmacy

Graduate School of Chosun University

A chalcone compound Isosalipurposide (ISPS) is successfully isolated from native Korean plant species *Corylopsis coreana Uyeki* (Korean winter hazel). However, the pharmacological role of ISPS is not elucidated yet even though it has been reported to have anti-microbial and anti-proliferative effects. This study investigated whether isosalipurposide has the capacity of Nrf2-antioxidant response element (ARE) signaling and induce its target gene expression, and to determine the protective role of ISPS against oxidative injury on hepatocytes. In HepG2 cells, nuclear translocation of Nrf2 is augmented by ISPS treatment. Consistently, ISPS increased ARE reporter gene activity and the protein levels of glutamate cysteine ligase (GCL) and hemeoxygenase-1(HO-1), resulting in increased intracellular glutathione levels. Pretreated with ISPS were significantly rescued from t-BHP-induced ROS production and glutathione depletion. Moreover, ISPS restored mitochondrial dysfunction and apoptosis. The specific role of Nrf2 in ISPS-induced Nrf2 activation

was demonstrated using an ARE-deletion mutant plasmid and Nrf2-knockout cells. Finally, we observed PKC, ERK and AMPK are involved in the activation of Nrf2 by ISPS. Taken together, our results demonstrate that ISPS has cytoprotective efficacy against oxidative damage by Nrf2 activation and inducing its target gene expressions in hepatocytes.