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Isorhamnetin protects against oxidative stress by activating Nrf2 and inducing the expression of its target genes

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ABSTRACT

Isorhamentin is a 3′-*O*-methylated metabolite of quercetin, and has been reported to have anti-inflammatory and anti-proliferative effects. However, the effects of isorhamnetin on Nrf2 activation and on the expressions of its downstream genes in hepatocytes have not been elucidated. Here, we investigated whether isorhamnetin has the ability to activate Nrf2 and induce phase II antioxidant enzyme expression, and to determine the protective role of isorhamnetin on oxidative injury in hepatocytes. In HepG2 cells, isorhamnetin increased the nuclear translocation of Nrf2 in a dose- and time-dependent manner, and consistently, increased antioxidant response element (ARE) reporter gene activity and the protein levels of hemeoxygenase (HO-1) and of glutamate cysteine ligase (GCL), which resulted in intracellular GSH level increases. The specific role of Nrf2 in isorhamnetin-induced Nrf2 target gene expression was verified using an ARE-deletion mutant plasmid and Nrf2-knockout MEF cells. Deletion of the ARE in the promoter region of the *sestrin2* gene, which is recently identified as the Nrf2 target gene by us, abolished the ability of isorhamnetin to increase luciferase activity. In addition, Nrf2 deficiency completely blocked the ability of isorhamnetin to induce HO-1 and GCL. Furthermore, isorhamnetin pretreatment blocked t-BHP-induced ROS production and reversed GSH depletion by t-BHP and consequently, due to reduced ROS levels, decreased t-BHP-induced cell death. In addition isorhamnetin increased ERK1/2, PKCδ and AMPK phosphorylation. Finally, we showed that Nrf2 deficiency blocked the ability of isorhamnetin to protect cells from injury induced by t-BHP. Taken together, our results demonstrate that isorhamnetin is efficacious in protecting hepatocytes against oxidative stress by Nrf2 activation and in inducing the expressions of its downstream genes.

Introduction

Excessive reactive oxygen species (ROS) production is believed to cause a diversity of diseases. Furthermore, balance between the generation and elimination of ROS keeps cellular homeostasis (Rodriguez and Redman, 2005). Under normal physiological conditions, cells control ROS levels using a sophisticated antioxidant defense mechanism. However, under pathological conditions, such as in chronic inflammatory conditions, excessive ROS causes cellular dysfunction and remodeling (Finkel, 2011; Valko et al., 2007).

NF-E2-related factor 2 (Nrf2) is a member of the cap'n'collar family of bZIP transcription factors that bind to the antioxidant response element (ARE) and thereby regulate induction of genes encoding antioxidant proteins and phase II detoxifying enzymes such as γ-glutamylcysteine synthetase (GCS) (Jeyapaul and Jaiswal, 2000), hemeoxygenase 1 (HO-1) (Kweon et al., 2006), glutathione *S*-transferase A1/2 (Kwak et al., 2001), NAD(P)H:quinone reductase (NQO1) (Venugopal and Jaiswal, 1996) and sestrin2 (Sesn2) (Shin et al., 2012). It has been well established that Nrf2 activation in response to oxidative injury protects cells and tissues from oxidative stress. Under normal conditions, Nrf2 is localized in the cytoplasm where it binds with the Keap1, which functions as an adaptor for Cul3-based E3 ligase to regulate the proteasomal degradation of Nrf2 (Venugopal and Jaiswal, 1996). In fact, the Nrf2–Keap1 interaction leads to the rapid degradation of Nrf2 via Cul3 ubiquitin E3 ligase polyubiquitination (Kobayashi et al., 2004). However, after direct attack by ROS or resulting indirect actions, such as phosphorylation, Nrf2 dissociates from Keap1 and thereby translocates into the nucleus and transactivates its target genes through ARE. 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

Abbreviations: Sesn2, sestrin2; ARE, antioxidant response element; t-BHQ, *tert*-butylhydroquinone; ROS, reactive oxygen species; Nrf2, NF-E2-related factor-2; GCS, γ-glutamylcysteine synthetase; HO-1, hemeoxygenase 1; NQO1, NAD(P)H:quinone reductase; ERK1/2, extracellular signal-regulated kinase; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; AMPK, 5′ AMP-activated protein kinase.

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and Mulcahy, 2003), protein kinase C delta (PKCδ) (Huang et al., 2002), phosphoinositide 3-kinase (PI3K) and 5′ AMP-activated protein kinase (AMPK) (Liu et al., 2011) in Nrf2 activation.

Nrf2 is ubiquitously expressed in a wide range of tissues, but is especially abundant in metabolic and detoxification organs, such as the liver (Lee et al., 2005). For this reason, the involvements of Nrf2 activation in the pathogenesis of liver diseases and its possible applications, as a therapeutic target to prevent and treat liver diseases have been extensively investigated (Shin et al., 2013). Actually, *in vivo* and *in vitro* studies have suggested that the Nrf2-ARE pathway exhibits diverse beneficial biological functions against viral hepatitis, alcoholic and non-alcoholic liver disease, fibrosis, and hepatocellular carcinoma (Bataille and Manautou, 2012; Shin et al., 2013). Furthermore, natural and synthetic compounds that modulate Nrf2 activity have been reported to be an effective strategy of new drug development for the liver diseases.

Isorhamnetin is an abundant flavonol aglycone in medicinal herbs and plants, such as the sea buckthorn (*Hippophae rhamnoides* L.) and water dropwort (*Oenanthe javanica*), which are frequently used in traditional medicine to prevent and treat diverse diseases (Park et al., 1995). Our laboratory recently succeeded to isolate isorhamnetin from the water dropwort and reported its anti-inflammatory effect against acute inflammation *in vivo* and *in vitro* (Yang et al., 2013). In addition, it has been reported to have anti-proliferative (Teng et al., 2006) and anti-viral (Apers et al., 2002) effects. Nevertheless, little is known about the effect of isorhamnetin on Nrf2 activation or the molecular mechanisms involved.

In this study, we investigated whether isorhamnetin has the capability to activate Nrf2 and to induce its downstream target genes in hepatocytes. In addition, the role of Nrf2 in isorhamnetin-induced Nrf2 target gene expression was verified by using an ARE deletion mutant plasmid construct and Nrf2 knockout MEF cells. Nrf2 activation by isorhamnetin was found to protect against oxidative injury and to have a cytoprotective effect in hepatocytes. Furthermore, Nrf2 activation by isorhamnetin was found to be due to the phosphorylations of ERK, PKCδ, and AMPK. Collectively, our results demonstrate that isorhamnetin protects hepatocytes against oxidative stress by activating Nrf2 and inducing phase II antioxidant gene expression, which might account for the pharmacological efficacy of isorhamnetin for liver diseases.

Materials and methods

Materials. 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, caspase-3, phospho-PKCδ, phospho-AMPK, phospho-ACC, tubulin and AMPK antibodies were obtained from Cell Signaling (Danvers, MA). Sesn2 antibody was obtained from Proteinteck (Chicago, IL) and GCL antibody from Abcam (Cambridge, MA). HO-1 antibody was provided by Enzo Life Sciences (Plymouth Meeting, PA) and MTT, 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).

Preparation of isorhamnetin. Isorhamnetin was isolated from *O. javanica* as previously reported (Yang et al., 2013). Briefly, 10 kg of air-dried stems and leaves of *O. javanica* were extracted three times with MeOH. The methanolic extract was then suspended in water and partitioned successively with $CHCl_3$ and *n*-BuOH. The *n*-BuOH fraction was loaded onto a silica gel column (15 × 80 cm, 70–230 mesh) and eluted initially with $CHCl_3$, and then with a $CHCl_3$-MeOH gradient. The $CHCl_3$-MeOH (25:1) fraction was concentrated to give a dark brown residue (23 g), which was further fractionated by silica gel column chromatography using *n*-hexane-EtOAc gradient. Fractions 35–40 from this column were combined and evaporated to give an isorhamnetin mixture (3 g). Finally, 2 g of purified isorhamnetin was obtained. Its chemical structure was confirmed by HPLC-ESI-MS and NMR spectroscopy as previously described (Yang et al., 2013).

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. Isorhamnetin or t-BHQ (30 μM), 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.

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 (Amersham Biosciences, Buckinghamshire, UK). Equal loading of proteins was verified by Coomassie blue staining of gels and β-actin, tubulin 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.

Plasmid construction and luciferase assay. The hSesn2 promoter-driven luciferase construct, pGL4-phSesn2, was kindly provided by RIKEN BRC through the National Bio-resource Project of MEXT, Japan. The 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 isorhamnetin. Transfected cells were incubated in DMEM containing 1% fetal bovine serum (FBS) (Hyclone, Logan, UT) for 3 h and exposed to isorhamnetin 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.

MTT cell viability 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 isorhamnetin (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).