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AMPK activation by isorhamnetin protects hepatocytes against oxidative stress and mitochondrial dysfunction

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ABSTRACT

Arachidonic acid (AA) is a $\omega-6$ polyunsaturated fatty acid that is found in the phospholipids of membranes and released from the cellular membrane lipid bilayer by phospholipase A2. During this process, AA could produce excess reactive oxygen species and induce apoptosis and mitochondrial dysfunction by selectively inhibiting complexes I and III. Isorhamnetin, an *O*-methylated flavonol aglycone, has been shown to have cardio-protective, anti-adipogenic, anti-tumor, and anti-inflammatory effects. In the present study, we investigated the effects of isorhamnetin on hepatotoxicity and the underlying mechanisms involved. Our *in vitro* experiments showed that isorhamnetin dose-dependently blocked the hepatotoxicity induced by treatment with AA plus iron in HepG2 cells. Furthermore, isorhamnetin inhibited the AA+iron induced generation of reactive oxygen species and reduction of glutathione, and subsequently maintained mitochondria membrane potential in AA+iron treated HepG2 cells. In addition, isorhamnetin activated AMP-activated protein kinase (AMPK) by Thr-172 phosphorylation of AMPKα, and this was mediated with Ca(2+)/calmodulin-dependent protein kinase kinase-2 (CaMKK2), but not liver kinase B1. Experiments using CaMKK2 siRNA or its selective inhibitor, STO-609, revealed the role of CaMKK2 in the isorhamnetin-induced activation of AMPK in HepG2 cells. These results indicate isorhamnetin protects against the hepatotoxic effect of AA plus iron, and suggest that the AMPK pathway is involved in the mechanism underlying the beneficial effect of isorhamnetin in the liver.

1. Introduction

Oxidative stress promotes cellular damage and is a characteristic feature of several human diseases. For example, it has been shown that modification of membrane phospholipids by excess reactive oxygen species results in cell and tissue injury (Apel and Hirt, 2004; Bergamini et al., 2004; Reddy and Clark, 2004; Shah and Channon, 2004; Valko et al., 2006; Willner, 2004). During conditions of oxidative and/or inflammatory stress, reactive oxygen species and/or cytokines can induce the oxidative modification of fatty acids within membrane phospholipids, and during this process, arachidonic acid (AA; a ω-6 polyunsaturated fatty acid) is released from the cellular membrane lipid bilayer (Balboa and Balsinde, 2006; Gijon and Leslie, 1999). AA can induce cell death by promoting the uptake of calcium by mitochondria and the production of ceramide (Balboa and Balsinde, 2006; Gijon and Leslie, 1999). In particular, in the presence of iron, released AA stimulates cells to produce more reactive oxygen species, which can induce mitochondrial dysfunction and cell death (Fleming and Bacon, 2005; Galaris and Pantopoulos, 2008; Halliday and Searle, 1996; Neufeld, 2006). Therefore, treatment with AA plus iron offers a treatment model that could be useful for screening agents that protect mitochondria against severe oxidative stress.

AMP-activated protein kinase (AMPK) is a multifunctional cytosolic protein that plays important roles in energy homeostasis,

Abbreviations: AA, arachidonic acid; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CaMKK2, Ca(2+)/calmodulin-dependent protein kinase kinase-2; DCFH-DA, 2′,7′-Dichlorofluorescein diacetate; FITC, fluorescein isothiocyanate; GSH, glutathione; IsoRN, Isorhamnetin; LKB1, liver kinase B1; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PI, propidium iodide.

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nutrient metabolism, cell survival, and apoptosis. Although AMPK is activated by cellular stresses including changes in the AMP/ATP ratio (Hardie and Carling, 1997), hypoxia (Sambandam and Lopaschuk, 2003), glucose deprivation (Xing et al., 2003) and osmotic stress (Tian et al., 2001), AMPK activators (e.g., metformin and dithiolethiones) increase cell survival by preventing changes in mitochondrial membrane potential (Detaille et al., 2005; Ido et al., 2002). Furthermore, some beneficial components and extracts of medical herbs protect cells against the severe oxidative stress induced by AA+iron by activating AMPK (Dong et al., 2013; Kim et al., 2009a; Shin and Kim, 2009).

In traditional Oriental medicine, water dropwort (*Oenanthe javanica*) is used to treat disorders in the lung, stomach and liver. Isorhamnetin (IsoRN, 3′-*O*-methyl quercetin) is an active flavonol aglycone found in the water dropwort, and has been reported to have multiple biological activities, which include cardioprotective, anti-adipogenic, and antitumor activities (Kim et al., 2011; Kong et al., 2009; Sun et al., 2012; Sun et al., 2013; Upadhyay et al., 2010; Zhang et al., 2011). Recently, we isolated IsoRN from the water dropwort and demonstrated its effects on acute inflammation *in vivo* and *in vitro* (Yang et al., 2013). In view of the importance of AMPK from the standpoint of cytoprotection and its potential as a cytoprotective, we hypothesized that IsoRN might protect against oxidative stress. Our work demonstrates that IsoRN protects cells against AA+iron induced apoptosis by inhibiting mitochondrial dysfunction and reactive oxygen species production. Furthermore, IsoRN was found by immunoblotting and immunoprecipitation analysis, to activate AMPK in the HepG2 cell line, which could explain in part the mitochondrial protective effect of IsoRN.

2. Materials and methods

2.1. Reagents

IsoRN was purified from water dropwort (*Oenanthe javanica*, *Umbelliferae*) using successive silica gel column chromatography, as previously described (Yang et al., 2013). The purity of the isolated IsoRN ($\geq$ 97%) was confirmed by ultra performance liquid chromatography analysis and the structures was verified on the basis of spectroscopic analyses including HPLC–ESI–MS (Agilent 6120 LC/MS system, Agilent Technologies, Palo Alto, CA) and NMR spectroscopy (data not shown), and compared with reported spectral data (Cao et al., 2009). Arachidonic acid (AA), compound C and STO-609 were purchased from Calbiochem (San Diego, CA, USA). Anti-procaspase-3, anti-phospho-acetyl-CoA carboxylase (ACC), anti-PARP and anti-phospho-AMPK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-AMPK, horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-goat, goat anti-mouse IgGs, CaMKK2 siRNA and liver kinase B1 (LKB1) siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ferric nitrate, nitrilotriacetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), rhodamine 123, 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), anti-β-actin antibody and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

HepG2 (human), AML12 (mouse), and H4IIE (rat) hepatocyte-derived cell lines were obtained from ATCC (Rockville, MD, USA). HepG2 and H4IIE were cultured in Eagle's minimum essential medium (DMEM) with 10% FBS. AML12 cells were cultured in DMEM/F12 medium. Cells were incubated in medium without 10% FBS for 12 h, and then stimulated with 10 μM AA for 12 h, followed by incubation to 5 μM iron. To determine the effects of IsoRN, the cells were treated with IsoRN for 1 h prior to the exposure with AA (Dong et al., 2013).

2.3. MTT assay

The MTT assay was performed as previously described (Dong et al., 2013). HepG2 cells were cultured in a 48-well plate with density of 1×10^5 cells/well. HepG2 cells were stained with 0.25 mg/ml MTT for 2 h after treatment of IsoRN or AA+iron or combination of AA+iron and IsoRN. The formazan crystals products were dissolved with the addition of 200 μl dimethylsulfoxide after remove media. Then, take 100 μl into 96-well plate and read the absorbance at 540 nm by ELISA microplate reader (Tecan, Research Triangle Park, NC, USA).

2.4. Annexin V and propidium iodide (PI) double staining

HepG2 cells were treated with IsoRN or AA+iron or combination of AA+iron and IsoRN, and then stained with fluorescein isothiocyanate (FITC) Annexin V and PI staining kit (Life Technology, Grand Island, USA) by manual. The 2×10^5 cells were counted by flow cytometer (FACS, Partec, Münster, Germany) and measured Fluorescence intensity of individual cell.

2.5. Immunoblot analysis

Lysis of cells and immunoblot analysis were performed as previously described (Dong et al., 2013). Cells were lysed in RIPA lysis buffer, and heated with SDS loading dye. The proteins were loaded onto SDS-PAGE gels, and transfer to NC membrane. The protein bands of interest were developed using an ECL chemiluminescence system (Amersham, Buckinghamshire, UK) after incubation with 1st and 2nd antibody.

2.6. Measurement of H_2O_2 production

The level of reactive oxygen species generation was determined by the concomitant increase in DCF fluorescence by a Fluorescence spectrometer (Partec, Münster, Germany). Cells were cultured in multi-well plates and treated with IsoRN or AA. After treatment, the cells were incubated with 10 μM DCFH-DA for 30 min, then incubated with iron for 1 h at 37 °C. Fluorescence intensity in the cells was measured using the Fluorescence spectrometer (Tecan, Research Triangle Park, NC, USA).

2.7. Determination of reduced GSH content

Glutathione (GSH) concentration in the HepG2 cells was measured by using a two-step chemical GSH #400 kit (Oxis International, Portland, OR, USA) (Dong et al., 2013). After treatments, cells were homogenized and measured the concentration of GSH by a spectrometer (Tecan, Research Triangle Park, NC, USA).

2.8. Flow cytometric analysis of mitochondrial membrane potential (MMP)

MMP was measured by FACS after staining with rhodamine 123, a membrane-permeable cationic fluorescent dye. After treatment of iron for 1 h, cells were stained with 0.05 μg/ml rhodamine 123 for 1 h, and harvested by trysinization. The change in MMP was measured the fluorescence intensity of cells by a FACS. In each analysis, 10,000 events were recorded.