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Morin hydrate ameliorates cisplatin-induced ER stress, inflammation and autophagy in HEK-293 cells and mice kidney via PARP-1 regulation

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ABSTRACT

The present study assessed the possible therapeutic potential of a natural flavonoid morin hydrate (MH), against cisplatin (CP) induced toxicity in HEK-293 cells and mice kidney. Herein, we observed that exposure of HEK-293 cells to CP (20 μM, 24 h) reduced the cell viability, and increased the intracellular ROS generation, nuclear DNA damage, Ca^{++} release, and accumulation of acidic vacuoles. Concomitantly, acute exposure of CP (30 mg/kg, 72 h) to male ICR mice induced histopathological changes in kidney tissue, and alterations in serum creatinine and blood urea nitrogen (BUN) levels. Oxidative stress mediated ER-stress was evidenced by the reduced expression of antioxidant enzymes such as SOD-1, SOD-2, GR, and Trx, and increased expression levels of CytP450, IRE1-α, PERK, and CHOP. The expression levels of major inflammatory response markers such as NF-κB, TNF-α, IL-1β, COX-2 and iNOS were significantly increased in the HEK-293 cells and mice kidney. Temporal up-regulation of p-AMPK and LC3I/II, and down regulation of mTOR was also noticed after CP treatment. CP-induced DNA damage led to activation of PARP-1, which plays a crucial role in inflammation, apoptosis and autophagy activation. Concurrently, co-treatment of CP-MH and CP-ANI (PARP-1 inhibitor) significantly attenuated the expression level of PARP-1, reduced cellular death, alleviated inflammatory responses, and inhibited autophagy stimulation in HEK-293 cells and mice kidney. On the basis of above findings, we suggest MH as a potential therapeutic agent against CP-induced nephrotoxicity.

1. Introduction

Cisplatin (CP) is an anti-neoplastic drug widely used in treating various solid organ cancers including those of the head and neck, ovarian, cervical and lung [1]. The mechanism of CP-induced anticancer therapy is widely thought to be interrelated with DNA cross-linking, resulting in the arrest of DNA synthesis and replication [2]. Although, CP is considered a mainstay in anticancer chemotherapy, its clinical significance is limited due to severe side effects on the normal body tissues and organs such as ototoxicity, neurotoxicity, gastric toxicity, myelosuppression and allergic reactions [3]. Since the main dose-limiting side effect of CP includes nephrotoxicity, this limits its use as a chemotherapeutic agent. A variety of approaches have been attempted to abolish the side effects, but no proper treatment is available to overcome the obstacle of CP chemotherapy [4]. CP nephrotoxicity is recognized as a complex process involving various signaling factors; research strategies to overcome this problem have focused on reducing the renal CP accumulation or metabolism and the anti-oxidant, anti-inflammatory and anti-apoptotic properties [5].

Previous studies have demonstrated that natural flavonoids like MH provide renoprotective activity against CP-induced renal damage via abrogation of oxidative stress and anti-apoptosis pathways [6]. However, other approaches which include reduction of ER-stress and autophagy by combined therapy of CP with natural medicinal compounds, is drawing a great deal of attention in past few years. Endoplasmic reticulum (ER) performs numerous cellular functions, such as regulation of protein biosynthesis, protein folding, trafficking, and modifications. Innumerable factors such as nutrient deprivation, temperature, and pH lead to disturbances of these functions, subsequently resulting in ER-stress [7]. ER-Stress can also be triggered by various intracellular

Abbreviations: AMPK, 5′ AMP-activated protein kinase; ANI, 4-Amino-1,8-naphthalimide; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein; COX-2, cyclooxygenase-2; ER, endoplasmic reticulum; eIF2-α, eukaryotic initiation factor 2-α; GR, glutathione reductase; GRP-78, glucose-regulated protein 78; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; iNOS, inducible nitric oxide synthase; IRE1-α, inositol-requiring enzyme 1α; JNK, c-Jun N-terminal kinases; LC3, microtubule-associated protein light chain; MPO, myeloperoxidase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PERK, protein kinase R-like endoplasmic reticulum kinase; PARP-1, poly (ADP-ribose) polymerase 1; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α; UPR, unfolded protein response

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stimuli, such as oxidative-stress, hyperhomocysteinemia, and disturbance in the Ca^{++} homeostasis [8,9]. Cells experiencing ER-stress invoke a well-conserved intracellular signaling known as unfolded protein response (UPR), which is classified into adaptive and maladaptive types. The adaptive UPR initially ensures cell survival by ameliorating the ER functional damage; however, if the ER-stress is severe or prolonged, the adaptive UPR fails to maintain the ER homeostasis [10]. ER-stress associated kidney diseases by the accumulation of misfolded proteins suggest its pathophysiological significance via expression of GRP-78 and the induction of apoptotic UPR activation facilitated by caspase-12 and JNK pathway [11]. At the same time, nuclear factor-κB (NF-κB), a transcriptional regulator of inflammatory-related genes, is activated by ER-stress via attenuation of the inhibitors of NF-κB, which is mediated by the PERK-eIF2α and IRE1α-JNK pathways [12].

Poly (ADP-ribose) polymerase 1 (PARP-1) is a nuclear enzyme that acts as a transcription factor to regulate the protein functions by poly (ADP-ribosyl)ation and gene expression. It catalyzes the transfer of multiple ADP-ribose molecules from nicotinamide adenine dinucleotide (NAD^+), and conjugates PAR onto various proteins, thereby leading to a variety of physiological processes including protein-protein interaction and modulation of protein functions. In addition, the role of PARP-1 as a transcriptional regulator is confirmed by using genetic or pharmacological inhibitors, demonstrating its influence on the expression of inflammatory genes, including NF-κB, TNF-α, IL-1β, IL-6, and TLR4 [13]. Moreover, ROS and DNA damage mediated PARP-1 activation, leading to mTOR inhibition, acts as a key player in autophagy induction [14]. Autophagy plays a major role in cell growth or development by maintaining the balance between synthesis, degradation and subsequent recycling of macromolecules and cell organelles [15]. Recent studies demonstrated that activation of autophagic cell death is widely dependent on lysosomal compartmentalization under prolonged cell stress conditions including ER stress [16].

Morin hydrate (2′,3,4′,5,7-pentahydroxyflavone; Fig. 1A), a flavonoid isolated from *Maclura pomifera*, *Maclura tinctoria* and from leaves of *Psidium guajava* possesses a broad array of pharmacological activities including antioxidant, anti-inflammatory, anti-apoptosis and anti-autophagy [17,19]. Several recent studies demonstrated the anti-apoptotic and the anti-inflammatory role of MH in response to chemical-induced renal damage via suppression of tumor necrosis factor α (TNF-α), interleukins, bax and casp-3 [18]. In our previous studies, we have reported the anti-oxidant, anti-autophagic and anti-inflammatory role of MH in RAW 264.7 cells and in murine liver [19,20]. However, the role of MH against CP-induced HEK-293 cells toxicity and mice kidney injury remains unclear. In the present study, we investigated the regulatory effect of MH against PARP-1 mediated ER-stress, inflammation and autophagy as a plausible mechanism to ameliorate the CP-induced nephrotoxicity.

2. Material and methods

2.1. Chemicals and reagents

Cisplatin (CP) (≥98% purity), DMEM, morin hydrate (MH), acridine orange, Hoechst-33342, 2′,7′-dichlorofluorescin diacetate (H_2DCFDA), Fura-2-acetoxymethyl ester (Fura 2-AM), Dithiothreitol (DTT), Torin, 4-amino-1,8-naphthalimide (ANI) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) were purchased from Sigma-Aldrich, St. Louis, USA. Antibodies were purchased from Abcam, Cell signaling, and Santa Cruz Biotechnology. Detailed list of antibodies is included in Supplementary Table S1.

2.2. Cell culture and in vitro treatment

HEK-293 (ATCC) human embryonic kidney epithelial cells were cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) in a CO_2 incubator (5% CO_2 in air) at 37 °C. Cells were either untreated or co-treated with CP (20 μM), and MH (10 and 20 μM) for 24 h.

2.3. Animals, experimental design and compound treatment

All animal experiments were approved by the Committee for Laboratory Animal Care (LMO No. LML-16-1134) and use of Daegu University. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Thirty-two, 5-week-old male ICR mice, each weighing 20 ± 5 g, were provided by Orient Bio Inc. (Seongnam, Korea). Animals were housed in an air-conditioned room at 22 ± 2 °C and 30 ± 10% relative humidity. They were fed ad libitum with normal chow and distilled water and observed under a 12 h light/12 h dark cycle for 7 days for quarantine and acclimation, in order to confirm there were no emerging abnormalities. After the quarantine period, animals were randomly divided into 4 groups, each consisting of eight mice. Treatment was for 3 consecutive days, at 24 h time interval: Group I (control): normal saline by intra-peritoneal injection (IP); Group II: CP (30 mg/kg, i.p.); Group III: MH (20 mg/kg, i.p.) after 1 h pre-treatment of CP (30 mg/kg, i.p.); and Group IV: MH (40 mg/kg, i.p.) after 1 h pre-treatment of CP (30 mg/kg, i.p.). All animals were sacrificed by cervical dislocation within 24 h after last treatment. The dose of CP (30 mg/kg) was selected based on its ability to induce alterations in the biochemical parameters of mice according to previous studies [21]. IP administration of compounds was resorted to maintain the long-term availability in body fluid. Whole blood of all mice were collected in K3 EDTA and SSGT vials (Soyagreentec Co. Ltd., Seoul, Korea) and serum was separated by centrifugation at 2500 × *g* for 15 min at 4 °C, after which it was stored at −80 °C till further analysis. The kidney tissues were immediately excised from the mice and pulverized in liquid nitrogen, and a small portion of kidney tissue was fixed in 4% formalin for histopathological analysis.

2.4. Cell viability assay

Cell viability was measured using the MTT bioassay, which provides a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflects the early cellular redox changes. Briefly, HEK-293 cells (1×10^5 cells/well) were seeded in each well of a 96-well plate and incubated with either CP (20 μM) alone, or combination of CP (20 μM)-MH (10–200 μM), for 24 h at 37 °C; this was followed by addition of MTT solution (5 mg/ml in PBS) for 4 h. The dark-blue formazan crystals formed in the cells were dissolved in DMSO, and the absorbance was measured at 540 nm with a microtitre plate reader (Bio-Tek Instrument Co., WA, USA).

2.5. Cell morphological assessment

Morphological changes in HEK-293 cells were assessed post-exposure to CP (20 μM) or CP (20 μM)-MH (10 and 20 μM) for 24 h at 37 °C. Cells were observed under a compound microscope at 200× magnification. (Nikon Eclipse TS200, Nikon Corp., Tokyo, Japan).

2.6. Intracellular ROS generation assay

To detect intracellular reactive oxygen species (ROS) production, HEK-293 cells were cultured on coverslips in 12-well plates and then treated with either CP (20 μM) or CP (20 μM)-MH (10 and 20 μM) for 24 h at 37 °C. After incubation, cells were rinsed with PBS (pH 8). The peroxide-sensitive fluorescent probe H_2DCFDA (10 μM) was added to each well and incubated for 30 min at 37 °C [22]. The ROS production was analyzed under a fluorescence microscope (Nikon Eclipse TS200, Nikon Corp., Tokyo, Japan) at 400× magnification.