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Research paper

Morin, a dietary flavonoid, exhibits anti-fibrotic effect and induces apoptosis of activated hepatic stellate cells by suppressing canonical NF-κB signaling

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ABSTRACT

In experimental liver fibrosis, activated hepatic stellate cells (HSCs) play a central role and thus, induction of apoptosis of activated HSCs is a promising therapeutic strategy for liver fibrosis. The present study was designed to elucidate the molecular mechanisms of the pro-apoptotic effects of morin, a dietary flavonoid, *in vitro* and *in vivo*. Culture-activated human HSCs (LX-2 cells) were treated with morin (50 μM) for 24 and 48 h, and the mechanism of cell death induced by morin was evaluated. Also, the anti-fibrotic and pro-apoptotic effect of morin in diethylnitrosamine (DEN)-induced fibrotic rats were determined. Morin induced apoptosis in cultured LX-2 cells by preventing the nuclear translocation of nuclear factor-κBp65 (NF-κBp65) by inhibiting NF-κB activation via inhibition of IκBα degradation and thereby suppressing anti-apoptotic proteins and activating caspases. In fibrotic rats, morin treatment resulted in inhibition of canonical NF-κB signaling and induction of apoptosis, mainly by downregulating Bcl-2, upregulating Bax and cyt c and by activation of caspase-9 and caspase-3. Translocation of phosphatidylserine to the outer membrane, altered nuclear morphology and DNA fragmentation confirmed the induction of apoptosis by morin. Overall, morin treatment ameliorated experimental liver fibrosis, most likely through induction of apoptosis by inhibiting canonical NF-κB signaling in activated HSCs. It is therefore postulated that morin is a potential therapeutic candidate for liver fibrosis.

1. Introduction

A majority of chronic liver diseases are associated with fibrosis and are generally initiated by a wide range of causative agents. Alcohol, viral hepatitis (HBV and HCV), biliary diseases, drug-induced liver injury, schistosomiasis, constant exposure to toxins and chemicals are some of the common causes of liver fibrosis [1,2]. Liver fibrosis is a reversible, progressive pathological process characterized by the accumulation of excess extracellular matrix (ECM) proteins. Hepatic stellate cells (HSCs) are quiescent, non-proliferative, vitamin-A storing cells, localized in the space of Disse that function as the principal storage sites of retinoids in normal liver. Upon activation, HSCs proliferate and undergo transdifferentiation from quiescent vitamin-A storing cells to activated myofibroblast-like cells secreting excess ECM proteins [3]. HSCs are the principal cell types involved in liver fibrogenesis [4] and survival of activated HSCs is the hallmark feature of liver fibrosis and inhibition of HSC activation or proliferation [5,6] and promotion of apoptosis of activated HSCs are some of the key aspects in anti-fibrotic drug research [7–9].

Nuclear factor-κB (NF-κB), the principal transcriptional regulator of inflammatory response is essential for liver cell survival and liver homeostasis [10,11]. Regulation of cell death, inflammation and wound healing not only emphasize the role of NF-κB in the progression of liver diseases, also highlights the mechanistic links between liver injury, inflammation, fibrosis and hepatocellular carcinoma [12]. It is reported that active NF-κB inhibits apoptosis of activated HSCs [13–16] and hence, when NF-κB activation is prevented or inhibited, the activated HSCs undergo enhanced apoptosis [9].

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Currently, immense consideration is bestowed on dietary flavonoids for their abilities in exerting anti-inflammatory, anti-fibrotic and anti-cancer effects, mainly by ameliorating oxidant stress, inhibiting cell proliferation, inducing cell cycle arrest and apoptosis [17]. Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone) is a bioflavonoid, isolated as a yellowish pigment, mainly from mill (*Prunus dulcis*), almond hull (*Psidium guajava L.*), fig (*Chlorophora tinctoria*), fruits and wine [18]. Morin has been reported to possess a variety of biological properties including the protection of cardiovascular cells [19], glomerular mesangial cells [20], hepatocytes [21,22], oligodendrocytes and neurons [23] against oxidative stress-induced damage. Also, morin exhibited anti-inflammatory [24], anti-fibrotic [25,26], anti-cancer [22,27,28], apoptosis inducing [29] and neuroprotective [30] effects.

An earlier study from this group elucidated the anti-fibrotic potential of morin in experimental liver fibrosis, where morin inhibited stellate cell proliferation by suppressing Wnt/β-catenin signaling [26]. However, the pro-apoptotic effect of morin on activated HSCs is still under scrutiny. Hence, the present study was designed to elucidate the apoptosis inducing efficacy of morin in LX-2 cells, *in vitro* and DEN induced fibrosis model, *in vivo*.

2. Methods

2.1. Chemicals and antibodies

Dulbecco's modified eagle medium – Glutamax (DMEM – Glutamax) and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY). Fast green FCF, Trypsin and antibiotic solution were procured from Himedia laboratories (Mumbai, India). Diethylnitrosamine (DEN), morin (purity-95%), diethylpyrocarbonate (DEPC), 4′, 6-diamidino-2-phenylindole (DAPI), 3, 3′-Diaminobenzidine (DAB), Sirius Red F3B (Direct Red 80), propidium iodide (PI), Hoechst 33258, Annexin-V/PI apoptosis assay kit and poly-L-lysine were purchased from Sigma Aldrich (St. Louis, MO, USA). Rabbit monoclonal NF-κBp65, mouse monoclonal IκBα, rabbit monoclonal caspase-9, rabbit monoclonal cleaved caspase-3, rabbit monoclonal β-actin, rabbit monoclonal Histone H3, anti-mouse and anti-rabbit Horseradish peroxidase (HRP) conjugated antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA). Mouse polyclonal α-smooth muscle actin (α-SMA), rabbit monoclonal Bax, mouse monoclonal Bcl-2, mouse monoclonal cyt c, anti-rabbit Alexa blue, anti-mouse Tetramethylrhodamine isothiocyanate (TRITC) red, anti-mouse Fluorescein isothiocyanate (FITC) green and anti-rabbit FITC green conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescense (ECL) detection system (SuperSignal® West Femto Maximum Sensitivity Substrate) was procured from Thermo Scientific Pierce, USA. Agarose, TRIzol reagent, random hexamer, deoxynucleotide triphosphates (dNTPs), moloney murine leukemia virus (M-MLV) reverse transcriptase enzyme and its buffer, PCR ready master mix (2X), DNA 100 bp ladder, agarose and bovine serum albumin (BSA) were purchased from GeNeiTM (Bangalore, India).

2.2. LX-2 cells and morin treatment

LX-2 cells obtained as a gift from Prof. S.L. Friedman (Mount Sinai School of Medicine), were maintained in DMEM medium supplemented with 2% FBS at 37 °C in a 5% CO_2 incubator [31]. 10 mM morin solution was prepared in DMSO and diluted with cell culture medium according to the experimental requirement. LX-2 cells were serum starved for 6 h before treatment with morin (50 μM), based on the concentration chosen from MTT results [26].

2.3. Immunocytochemistry

Cells were immunostained with anti-α-SMA, anti-NF-κBp65, anti-Bax, anti-Bcl-2, anti-cyt c and visualized with a fluorescence microscope (Axioskope 2d, Carl Zeiss, Germany) as described earlier [26].

2.4. Flow-cytometric quantification of apoptosis

Cells were immunostained with annexin V-FITC/PI, analyzed by flow cytometry according to manufacturer's protocol (Sigma Aldrich). Only green fluorescein-positive cells without PI staining were regarded as apoptotic cells.

2.5. Assessment of apoptotic cell nuclear morphology

Cells were stained with Hoechst 33258 solution for 10 min and the nuclear morphology of apoptotic cells with condensed/fragmented nuclei were examined using a fluorescence microscope.

2.6. Animals and ethics statement

Male Wistar rats (*Rattus norvegicus*) of albino strain, weighing about 200 g, obtained from Kings Institute of Preventive Medicine (Chennai, India) were fed with standard pellet diet (Hindustan Lever Ltd., Bangalore) and water, *ad libitum*. All experimental procedures were approved by the institutional ethical committee for the use of small animals in biomedical research at University of Madras, Chennai (IAEC No.01/080/09).

2.7. Liver fibrosis and morin treatment

Liver fibrosis was induced by intraperitoneal injection of DEN (100 mg/kg body weight (BW)), once a week for six weeks and the fibrotic rats were treated with morin (50 mg/kg BW), orally, as described earlier [26]. Rats were randomly divided into four groups, each group consisting of six rats as follows: Group I (Control) rats received normal diet and pure drinking water. Group II (Morin control) rats received morin (50 mg/kg BW), orally, thrice a week, for six weeks. Group III (DEN induced) rats received intraperitoneal injection of DEN (100 mg/kg BW), once a week for six weeks. Group IV (Morin treated) rats received DEN as in Group III and morin as in Group II for six weeks.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRI reagent and cDNA of each sample was synthesized by reverse transcription method as described earlier [26]. The primers used in PCR experiments were purchased from Sigma Aldrich. PCR was carried out using the following primers: human α-SMA (sense 5′-ctgttccagccatccttcat-3′, antisense 5′-gctggaaggtggacagagag-3′); human NF-κBp65 (sense 5′-ggactgccgggatggcttctat-3′, antisense 5′-gctgctcttcttggaaggggttgt-3′); human Bcl-2 (sense 5′-agatgtccagccagctgcacctgac-3′, antisense 5′-agataggcacccagggtgatgcaagct-3′); human Bax (sense 5′-tgcttcagggtttcatccag-3′, antisense 5′-ggcggcaatcatcctctg-3′); human β-actin (sense 5′-gatgagattggcatggcttt-3′, antisense 5′-gagaagtggggtggctt-3′); rat α-SMA (sense 5′-tgctccagctatgtgtgaagaggaa-3′, antisense 5′-cctctcttgctctgcgcttcgt-3′); rat NF-κBp65 (sense 5′-acgatctgtttccccctcatct-3′, antisense 5′-tgcttctctccccaggaata-3′); rat TNF-α (sense 5′-agatgtggaactggcagagg-3′, antisense 5′-cccatttgggaacttctcct-3′); rat cox-2 (sense 5′-aaagcctcgtccagatgcta-3′, antisense 5′-atggtggctgtcttggtagg-3′); rat IL-6 (sense 5′-ccggagaggagacttcacag-3′, antisense 5′-acagtgcatcatcgctgttc-3′); rat GAPDH (sense 5′-tcaagaaggtggtgaagcag-3′, antisense 5′-