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Research Paper

# Suppression in PHLPP2 induction by morin promotes Nrf2-regulated cellular defenses against oxidative injury to primary rat hepatocytes

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ABSTRACT

Recent advances indicate a possible role of phytochemicals as modulatory factors in signaling pathways. We have previously demonstrated PHLPP2-mediated suppression of Nrf2 responses during oxidant attack. The present study was designed to explore Nrf2-potentiating mechanism of morin, a flavonol, via its possible role in intervening PHLPP2-regulated Akt/GSK3β/Fyn kinase axis. Efficacy of morin was evaluated against oxidative stress-mediated damage to primary hepatocytes by tert-butyl hydroperoxide (tBHP) and acetaminophen. The anti-cytotoxic effects of morin were found to be a consequence of fortification of Nrf2-regulated antioxidant defenses since morin failed to sustain activities of redox enzyme in Nrf2 silenced hepatocytes. Morin promoted Nrf2 stability and its nuclear retention by possibly modulating PHLPP2 activity which subdues cellular Nrf2 responses by activating Fyn kinase. Pull-down assay using morin-conjugated beads indicated the binding affinity of morin towards PHLPP2. Molecular docking also revealed the propensity of morin to occupy the active site of PHLPP2 enzyme. Thus, dietary phytochemical morin was observed to counteract oxidant-induced hepatocellular damage by promoting Nrf2-regulated transcriptional induction. The findings support the novel role of morin in potentiating Nrf2 responses by limiting PHLPP2 and hence Fyn kinase activation. Therefore, morin may be exploited in developing novel therapeutic strategy aimed at enhancing Nrf2 responses.



## 1. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) have the capacity to act as signaling moieties involved in maintenance of cellular homeostasis [1]. However, their accumulation within the cell may lead to oxidative imbalances, altered homeostasis and subsequent cell death. Such an occurrence is prevented through a coordinated defense network, such as the Antioxidant Response Element (ARE)/Nrf2 pathway, that guards the cellular makeup from a possible oxidative insult. When confronted with oxidant challenge, Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), translocates to the nucleus, where, in association with other proteins, it keeps a panel of cytoprotective genes under its tight regulation [2]. Nrf2 is activated and translocated to the nucleus even when a mild rise in oxidative load is sensed [3]. However, any irregularity in its mechanism potentiates free radical-induced stress, thereby disturbing normal cellular physiology.

Perturbed Nrf2 activity has been associated with the progression of a number of pathological conditions involving oxidative imbalances [4–6]. Our previous findings on oxidative toxicity imposed by *tert*-butyl hydroperoxide (tBHP) [7] documented suppression in cellular Nrf2 responses due to induction of PH domain Leucine-rich repeat Protein Phosphatase 2 (PHLPP2) which

*Abbreviations:* AIF, apoptosis inducing factor; APAP, acetaminophen; ARE, antioxidant redox element; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DHE, dihydroethidium; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; HO1, heme oxygenase 1; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide; Keap1, Kelch-like ECH-associated protein-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO1, NAD(P)H quinine oxidoreductase 1; Nrf2, Nuclear factor erythroid 2-related factor 2; PHLPP2, PH-domain and Leucine rich repeat protein phosphatase 2; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SEM, standard error of the mean; siRNA, small interfering RNA; tBHP, tert-butyl hydroperoxide; TrxRed, thioredoxin reductase

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ultimately led to GSK3β and Fyn kinase activation. PHLPP isozymes (PHLPP1 and PHLPP2) regulate the PI3K signaling by selective dephosphorylation (of Akt Ser473 residue) and hence down-modulation of Akt activity [8]. The phosphatidylinositol-3 kinase (PI3K) signaling is well known to play a pivotal role in maintaining cellular homeostasis. Due to its central role in deactivation of Akt, PHLPP is viewed as an attractive drug target for positive or negative regulation of PI3K signaling in disease [9]. Owing to the inhibitory effect of PHLPP2 on Nrf2 activity [7], checking PHLPP2 activity may prove beneficial under circumstances where Nrf2 signaling is muted due to increased Nrf2 destabilization.

The functional integrity of Nrf2-regulated antioxidant and detoxification system is of utmost importance for the maintenance of hepatic physiology. At present, dietary phytoconstituents that could assist in alleviating oxidative stress-associated repercussions are increasingly being sought-after [10,11]. Plant-derived phytochemicals are now widely accepted as important dietary factors that boost our health by protecting cells against oxidative damage. The wide range of health promoting effects of these phytochemicals has often been attributed to the induction of Nrf2 pathway [12]. Morin (2′,3,4′,5,7-pentahydroxyflavone), a flavonol, has been shown to possess a wide array of biological activities including anti-oxidant [13], anti-hyperglycaemic [14] and hepato-protective [15] properties. Though its role as an antioxidant or free-radical scavenger in promoting cytoprotection has been established, no study has yet addressed its function as modulator of Nrf2-mediated signaling pathways to counter cytotoxicity arising due to oxidative stress.

Hence, the present study was aimed at defining the mechanistic involvement of morin in mitigation of dysregulated Nrf2 responses during an event of oxidant attack by employing tBHP as an oxidative stress generating agent. Further, the cytoprotective mechanism of morin investigated in oxidatively compromised (tBHP-treated) hepatocytes was also confirmed in hepatocytes exposed to cytotoxic concentrations of acetaminophen (APAP).

## 2. Materials and methods

### 2.1. Materials and reagents

Antibodies and chemicals including ERK1/2 (4695), phospho-ERK1/2 (9101), SAPK/JNK (9252), phospho-SAPK/JNK (4668), phospho-Akt Ser473 (4060), phospho-Akt Thr308 (2965), phospho-GSK3β Ser9 (9323), phospho-PDK1 Ser241 (3438), phospho-PTEN Ser380 (9551), Cox-IV (11967), anti-rabbit Alexafluor 555 conjugate (4413) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against caspase-12 (ab62484), HMGB1 (ab79823) and NALP3 (ab17267) were obtained from Abcam (Cambridge, UK). FBS, 100X antimycotic and antibiotic solution, Collagenase (type IV), OPTI-MEM reduced serum medium, William's medium E, Lipofectamine RNAiMAX Transfection Reagent, CellTracker™ Green CMFDA dye and antibody against caspase-1 (AHZ0082) were procured from Invitrogen (Carlsbad, CA). Silencer Select Pre-designed siRNA against Nfe2l2 (ID:s136127), negative control unlabeled siRNA were supplied by Ambion (Austin, TX). Chemicals and antibodies like Nrf2 (sc-30915), Keap1 (sc-33569), β-actin (sc-81178), GAPDH(sc-25778), Ubiquitin (sc-166553), Lamin b (sc-6216), HO1 (sc-10789), NQO1 (sc-16463), Akt1 (sc-5298), Fyn kinase (sc-16-G), caspase-3 (sc-7148), caspase-9 (sc-8355), Bax (sc-493), Bcl2 (sc-492-G), cytochrome c (sc-8385), agarose conjugated phospho-Thr antibody (sc-5267AC), secondary antibodies, normal goat sera and Protein A/G PLUS Sepharose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CNBr-activated Sepharose beads were purchased from GE-Healthcare (GE Healthcare BioSciences Ltd., Kowloon, Hongkong). All other reagents or chemicals including acetaminophen, morin hydrate, tBHP, PHLPP2 antibody (SAB1300919), anti-goat FITC conjugated secondary antibody (F7367), Hoechst 33258 and dexamethasone were procured from Sigma (St Louis, MO, USA) unless otherwise mentioned.

### 2.2. Primary rat hepatocytes isolation, culture and treatment

Primary hepatocytes were isolated from male wistar rats weighing 100–120 g, 6- to 8-week-old through portal vein collagenase perfusion of liver as per the method of Seglen [16]. Rats were procured from the animal house of CSIR-Indian Institute of Toxicology Research. All rats were housed in environmentally controlled rooms under standard conditions of humidity 60–70%, temperature 25 ± 2 °C and a 12 h light/dark cycle. Animal handling in all experimental procedures was approved by the Institutional Animal Ethics Committee (Ref no. ITRC/IAEC/20/09-01/11-33/12). Hepatocytes were seeded on collagen-coated surface and were cultured for 4 h in William's medium E supplemented with 50 nmol/l dexamethasone and 5% fetal bovine serum (FBS) in addition to 2 mmol/l glutamine. Thereafter, the cells were cultured in the same medium lacking dexamethasone and FBS. tBHP was freshly prepared in culture medium while morin stock solution was prepared in dimethyl sulfoxide (DMSO) and thereafter diluted in culture medium taking care that DMSO concentration does not exceed 0.01% in any of the treatments. Cytoprotective efficacy of morin against tBHP was tested by treating hepatocytes with different concentrations of morin in combination with tBHP stress. In case of morin pre-treatment, hepatocytes were exposed to selected concentration of 10 μM morin for different time periods and washed with fresh media before subsequent treatment with tBHP. In experiments involving acetaminophen (APAP), hepatocytes were treated with 1 mM APAP (prepared by dissolving in media) for 90 min. A concentration of 10 μM morin was tested for its efficacy against APAP. Cell viability was estimated by determining the reduction of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. The absorbance corresponding to that of untreated control cells was assumed as 100% cell viability.

### 2.3. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS generation was estimated using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Briefly, hepatocytes plated at a density of 10,000 cells/well were incubated with DCFH-DA (10 μM) for 30 min at 37 °C prior to treatment with tBHP and/or morin. In case of morin pre-treatment, DCFH-DA (10 μM) was added 30 min prior to end of 3 h incubation with morin. Measurements were taken at different time periods during the course of the treatment schedule using Varioskan Flash Multimode microplate reader (Thermo Fisher Scientific) at 485 nm excitation and 530 nm emission.

### 2.4. Measurement of glutathione (GSH) levels

For glutathione estimation, CellTracker™ Green CMFDA dye (5-Chloromethylfluorescein Diacetate; Invitrogen) was used, which detects GSH with a specificity of 95%. Cells were incubated with 5 μM CMFDA at 37 °C prior to treatment and measurements were made at different time periods at excitation wavelength of 492 nm and emission wavelength of 517 nm using Varioskan Flash Multimode microplate reader (Thermo Fisher Scientific).

### 2.5. Evaluation of mitochondrial membrane potential

Cells were treated with 5 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-