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Morin modulates the oxidative stress-induced NF-κB pathway through its anti-oxidant activity

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Abstract

Morin is a flavone that has anti-inflammatory effects through a mechanism that is not well understood. Based on the extreme sensitive nature of the transcription factor, NF-κB to redox change, it is postulated that morin's anti-NF-κB activation likely depends on its ability to scavenge excessive reactive species [RS]. The present study assessed the extent of morin's ability to modulate RS-induced NF-κB activation through its scavenging activity. Results indicate that morin neutralized RS *in vitro* and inhibited *t*-BHP-induced RS generation. It also examined morin for suppressed redox-sensitive transcription factor NF-κB activation via reduced DNA binding activity, IκBα phosphorylation and p65/p50 nuclear translocation. The more important finding was that suppression of the NF-κB cascade by morin was modulated through the ERK and p38 MAPKs signal transduction pathways in endothelial cells. As a consequence, morin's anti-oxidant effect extended expression level of NF-κB dependent pro-inflammatory genes, thereby reducing COX-2, iNOS and 5-LOX. The data indicate that morin has strong anti-oxidative power against RS-induced NF-κB modulation through the ERK and p38 MAPKs signalling pathways by its RS scavenging activity. The significance of the current study is the new revelation that morin may have potential as an effective anti-inflammatory therapeutic agent.

Keywords: *Morin, reactive species, oxidative stress, NF-κB, MAPKs, anti-inflammatory, RS scavenger, flavones*

Introduction

Flavonoids from natural products are known to produce beneficial biological and pharmacological effects against oxidative stress-related diseases such as cancer, ageing and diabetes. Flavonoids have been reported as potent antioxidants to reduce risk of many chronic diseases [1,2]; therefore, it is important to investigate the effect of flavonoids for the prevention of RS-associated disorders.

Morin (2′,4′,3,5,7-pentahydroxyflavonoid) is among the members of the *Moraceae* family (Figure 1), such as mulberry figs and almond hulls (*Chlorophora tinctoria*). Over the years, numerous activities have been assigned to morin. Morin is one such dietary bioflavonoid that is reported to produce anti-oxidative [3], anti-inflammatory and anti-carcinogenic effects [4–10]. The ability of morin to protect against oxidative stress-induced damage [11,12] may stem from its action against reactive oxygen species (RS) such as superoxide anions, hydroxyl radicals, hydrogen peroxides and other highly reactive molecules [13]. RS generated in the body have been reported to be associated with damage to cells and this damage induces various deleterious biological effects including inflammation, cell ageing and cell death.

Reactive species play a major role in activating inflammatory responses through nuclear factor-κB

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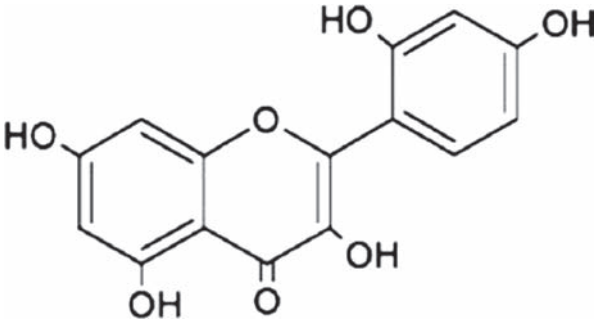

Figure 1. Chemical structure of morin (2′,4′,3,5,7-pentahydroxyflavonoid).

(NF-κB) under normal conditions. NF-κB is a heterodimeric protein complex made of sub-units of both p50 and p65 and is induced by various inflammatory stimuli like growth factors and cytokines. NF-κB is retained in the cytoplasm by the inhibitory sub-unit, IκBα [14], which binds to a member of the IκBα inhibitor protein family in an inactive state, and following stimulation, IκBα is phosphorylated and then degraded. Unbound NF-κB translocates into the nucleus and transactivates various downstream genes. In its unstimulated form, NF-κB is activated by a wide variety of inflammatory stimuli, including TNF, IL-1, H_2O_2, LPS and γ-radiation.

Upon activation, NF-κB regulates the expression of pro-inflammatory genes, which include enzymes (COX-2 and iNOS), cytokines (TNF, IL-1, IL-6, IL-8 and chemokines), adhesion molecules (VCAM-1 and ICAM-1), cell cycle regulatory molecules and angiogenic factors [15]. MAPKs are proline-directed serine/threonine protein kinases that are regulated various stimuli and are important mediators involved in cellular responses. Three distinct mitogen-activated protein kinase (MAPK) sub-families have been identified in mammalian cells: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAPK, which typically affect the activation of NF-κB [16]. A previous study showed NF-κB activation by oxidative stress, indicating an alternate signalling pathway [17]. Activation of p38 MAPK by various extracellular stimuli also has been shown to lead to the activation of NF-κB [18]. Morin is shown to modulate several pro-inflammatory gene products that are regulated by NF-κB [19,20], but its mechanisms are unclear.

In the present study, we tested our hypothesis that morin modulates redox-sensitive transcription factor NF-κB activation via the ERK and p38 MAPKs signalling pathways by its RS scavenging activity. We used *in vitro* models and endothelial cells, finding that morin showed strong antioxidant activity by suppressing RS-induced NF-κB activation through the inhibition of IκB phosphorylation by modulate ERK, p38 MAPKs pathways.

Material and methods

Reagents

Morin (3,5,7,2′,4′-Pentahydroxyflavone), SIN-1 (3-Morpholinosydnonimine hydrochloride), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and tert-butylhydroperoxide (*t*-BHP) were obtained from Sigma Chemical Co. (St. Louis, MO). 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) was purchased from Molecular Probes (Eugene, OR). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Corp. (Billelica, MA) and the chemiluminescence detection system was obtained from Amersham Life Sciences, Inc. (Arlington Heights, IL). Antibodies to p65, p50, p-IκB, IκB, p-p38, p-ERK, p-JNK, COX-2, iNOS, 5-LOX, TFIIB and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham Life Science (Buckinghamshire, UK). All other chemicals were of the highest purity available from either Sigma Chemical Co. (St. Louis, MO) or Junsei Chemical Co. (Tokyo, Japan).

Cell culture conditions and treatments

YPEN-1 cells, rat endothelial cells, were obtained from ATCC (American Type Culture Collection, Rockville, MD). YPEN-1 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Nissui Co., Tokyo, Japan) supplemented with 5% heat-inactivated (56°C for 30 min) foetal bovine serum (FBS, Sigma), 233.6 mg/mL glutamine, 100 μg/mL penicillin-streptomycin and 0.25 μg/mL amphotericin B and adjusted to pH 7.4–7.6 with $NaHCO_3$ in an atmosphere of 5% CO_2. The fresh medium was replaced after 1 day to remove non-adherent cells or cell debris. A commercial *t*-BHP (70% aqueous solution) was obtained from Sigma Inc. Working solutions of *t*-BHP were made in phosphate buffered saline (PBS) immediately before use. For all experiments, cells were plated in 100 mm culture dishes and cultures at 70–80% of confluences were used for the chemical exposures. After a 24-h attachment period, media were replaced with serum free media and cells were treated with 20 μM *t*-BHP and afterwards were pre-incubated for 30 min with morin. After 30 min, 1 or 4 hperiods, cells were harvested with ice-cold PBS. Cell lysates were used for the Western blot analysis.

RS scavenging activity assay

RS scavenging was measured by monitoring the oxidation of 2′,7′-dichlorofluorescin diacetate (DCF-DA) by modifying the previous reported method [21]. The RS scavenging ability via DCF-DA was

measured at room temperature on a microplate. RS induced by SIN-1 and trolox was used as a positive control. The fluorescence intensity of DCF was measured for 30 min by using microplate fluorescence with excitation and emission wavelengths of 485 and 535 nm, respectively.

Measurement of intracellular RS levels

The cells inoculated at a density of 3×10^4cells/well in a 96-well plate were allowed to adhere overnight and then cells were incubated in serum free DMEM with *t*-BHP and/or morin and 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA, Molecular Probes) at 37°C. The change in fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm by Microplate Reader FL500 (Bio-Tek Instruments, Winooski, VT). A fluorometric assay was performed to determine the relative levels of RS, such as superoxide radical, hydroxyl radical and hydrogen peroxide [21]. This assay measures the oxidative conversion of stable, non-fluorescent DCF-DA to the highly fluorescent dichlorofluorescein (DCF) in the presence of esterases and reactive species [22]. This probe has been used extensively for quantification of RS and lipid hydroperoxides after careful examination for potential pitfalls in assay conditions [23].

Preparation of cytosolic and nuclear fraction

Nuclear and cytosolic extracts were prepared according to Deng et al. [24]. Briefly, treated cells were washed and then scraped into 1.0 ml of ice-cold PBS and centrifuged at 3000 rpm at 4°C for 5 min. The pellets were suspended in 10 mM Tris (pH 8.0) with 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40 (NP-40) and inhibitors incubated on ice for 15 min. Nuclei were separated from cytosol by centrifugation at 12 000 rpm at 4°C for 15 min. The cytosolic supernatants were removed and the precipitated pellets were suspended in 10 mM Tris (pH 8.0), with 50 mM KCl, 100 mM NaCl and inhibitors, incubated on ice for 1 h then they were centrifuged at 12 000 rpm at 4°C for another 30 min.

Analysis of proteins by Western blot

Western blotting was carried out as described previously [25]. The cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS) and lysed in buffer (50 mM Tris-HCl, pH 8.0, 120 mM sodium chloride, 0.5% Nonidet P-40 (NP-40) that was supplemented with protease and phosphatase inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 0.1 mM sodium orthovanadate and 50 mM sodium fluoride) for 1 hon ice, vortexing every 5 min. Lysates were centrifuged at 12 000 × g for 30 min to remove insoluble material. The protein concentration was determined by the Lowry's method using bovine serum albumin (BSA) as a standard. Equal amounts of protein were separated on 10–12% SDS-PAGE gels. The gels were subsequently transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA) by electroblotting for 2 h at 60–75 V. The membranes were blocked in a 5% non-fat milk solution in TBS with 0.5% Tween-20 and incubated with primary antibodies as indicated. Pre-stained blue protein markers (Bio-Rad) were used for molecular-weight determination.

Luciferase reporter gene assay

NF-κB activity was examined using a luciferase plasmid DNA, pTAL-NF-κB, that contains a specific binding sequence for NF-κB (Becton Dickinson (BD) Biosciences, Franklin Lakes, NJ). Transient transfection was carried out using FuGene 6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacture's instructions. After transfection, cells were treated with reagents per the experimental design. Briefly, YPEN-1 cells were seeded into 48-well plates (1×10^5 cells/mL and 250 μL per well) and cultured in DMEM containing 10% FBS overnight. For transfection, the cells should be more than 50% confluent. For single transfection, plasmid (0.1 μg per well) was used and for cotransfection, plasmids were mixed in a 1:1 ratio to a total amount of 0.1 μg/well. Following transfection, cells were cultured for 24 h and then exposed to DMEM containing 1% FBS with/without designated reagents for an additional 8 h. Luciferase activity was measured with the Steady-Glo Luciferase Assay System (Promega) and detected by luminometer GENios Plus (Tecan Group Ltd, Salzburg, Austria). The obtained raw luciferase activities were normalized by protein concentration per well.

Statistical analysis

ANOVA was conducted to analyse significant differences among all groups. Differences among the mean of individual groups were assessed by the Fischer's Protected LSD post-hoc test. Values of $p < 0.05$ were considered statistically significant.

Results

Scavenging ability of morin on SIN-1-induced oxidative stress

The ability of morin to scavenge RS was investigated using DCFDA, as shown in Figure 2. SIN-1 is the donor RS and potential RS generator that is

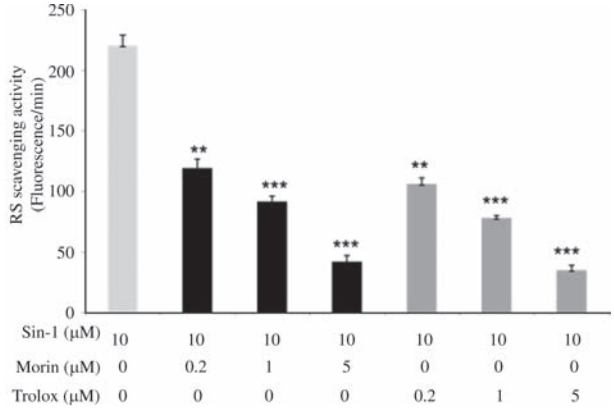

Figure 2. RS scavenging ability of morin on SIN-1-induced oxidative stress. The ability of morin to scavenge RS was investigated using DCF-DA, as shown in the Material and methods section. SIN-1 is the donor RS and potential RS generator. The scavenging activity using florescence intensity was measured for 30 min. The results show that morin has strongly SIN-1-induced RS scavenging activity in a dose-dependent manner compared with the positive control, trolox. Morin inhibited more than 80% of SIN-1-induced RS scavenging activity at 5 μM. $^{**}p < 0.01$ vs control.

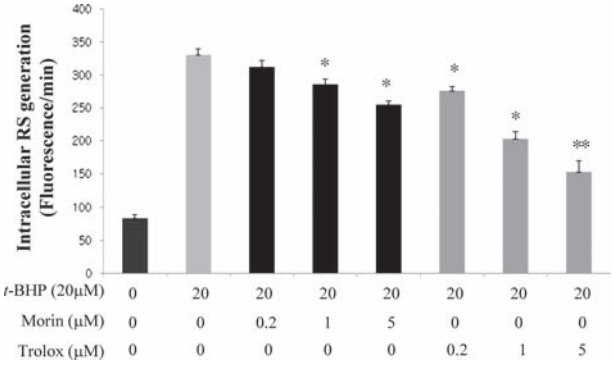

Figure 3. Effects of morin on *t*-BHP-induced RS generation in YPEN-1 cells. The formation of RS after 30 min of *t*-BHP exposure was detected using DCF-DA oxidation and fluorescence in the pre-treatment of morin (0.2, 1 or 5 μM) for 2 h. YPEN-1 cells were cultured for 12 h in standard DMEM medium containing 10% FBS. The rate of DCF formation was then evaluated by fluorescence intensity. $^{###}p < 0.001$ vs control, $^{*}p < 0.05$, $^{**}p < 0.01$ vs the group treated by only 20 μM *t*-BHP.

well-known to induce RS. Florescence intensity was measured for 30 min for scavenging activity. The results showed that morin has strong anti-oxidant activity to SIN-1-induced RS in a dose-dependent manner compared with positive control, trolox. Morin inhibited more than 80% of SIN-1-induced RS scavenging activity at 5 μM.

Inhibitory effect of morin on t-*BHP-induced RS production*

To determine the anti-oxidant effect of the morin *in vitro* cell system, RS generation was detected in *t*-BHP-treated cells. Thus, we determined the effects of morin on *t*-BHP-induced intracellular RS generation in YPEN-1 cells using DCF-DA, which is oxidized by RS to fluorescence DCF. YPEN-1 cells were seeded in a 96-well plate. After 1 day, the medium was changed to a fresh serum-free medium. The cells were treated with or without morin pre-incubated for 1 h. After treatment with 20 μM *t*-BHP for 30 min, the medium was replaced with a fresh serum-free medium and DCFDA (2.5 μM) was added. Intracellular RS formation resulting from *t*-BHP exposure was significantly reduced when present in the medium. When comparing cells treated with *t*-BHP against those pre-treated with morin (0.2, 1 or 5 μM), we found the increased RS was clearly suppressed in a dose-dependent manner (Figure 3). The results showed that morin has an intracellular capacity to prevent oxidative stress.

Suppressive effect of morin on t-*BHP-induced NF-κB activation*

To determine the suppressive effect of NF-κB activation by morin in endothelial cells, we examined p65 and p50 translocation to nucleus and IκB phosphorylation by Western blot using p65, p50, IκB and phosphorylated IκB-specific antibodies. As shown in Figure 4A, nuclear translocation of p65 and p65 significantly increased in *t*-BHP-stimulated nuclear lysate, while the morin-treated group showed lower levels of p65 and p65. *t*-BHP induced phosphorylation and degradation of IκBα in the endothelial cells. Morin also decreased phosphorylation and degradation of IκBα compared with *t*-BHP-stimulated cytosol lysate. These data indicate that morin modulated t-BHP-exposed NF-κB activation in endothelial cell.

Suppressive effect of morin on t-*BHP-induced NF-κB binding activity*

To investigate the inhibitory effect of morin on NF-κB activity, we used luciferase plasmid DNA, pTAL-NF-κB, that contains a specific binding sequence for NF-κB transiently transfected YPEN-1 cells. Luciferase activity was detected by RS treatment in the absence or presence of a morin after transient transfection of a plasmid containing NF-κB consensus and reporter. NF-κB luciferase activity increased more than 2-fold in cells exposed to 20 μM of *t*-BHP for 6 h. Treatment of YPEN-1 cells with 20 μM of *t*-BHP markedly enhanced NF-κB luciferase activity. However, the increase of NF-κB luciferase activity was significantly suppressed by the addition of morin in a dose-dependent manner (Figure 4B). These data suggest that morin might play a pivotal role in maintaining redox balance and modulating inflammatory reactions.

Modulation of morin on t-*BHP-induced phosphorylation of ERK and p38 MAPKs*

It is known that phosphorylation of MAPKs is modulated by *t*-BHP-induced oxidative stress. Therefore,

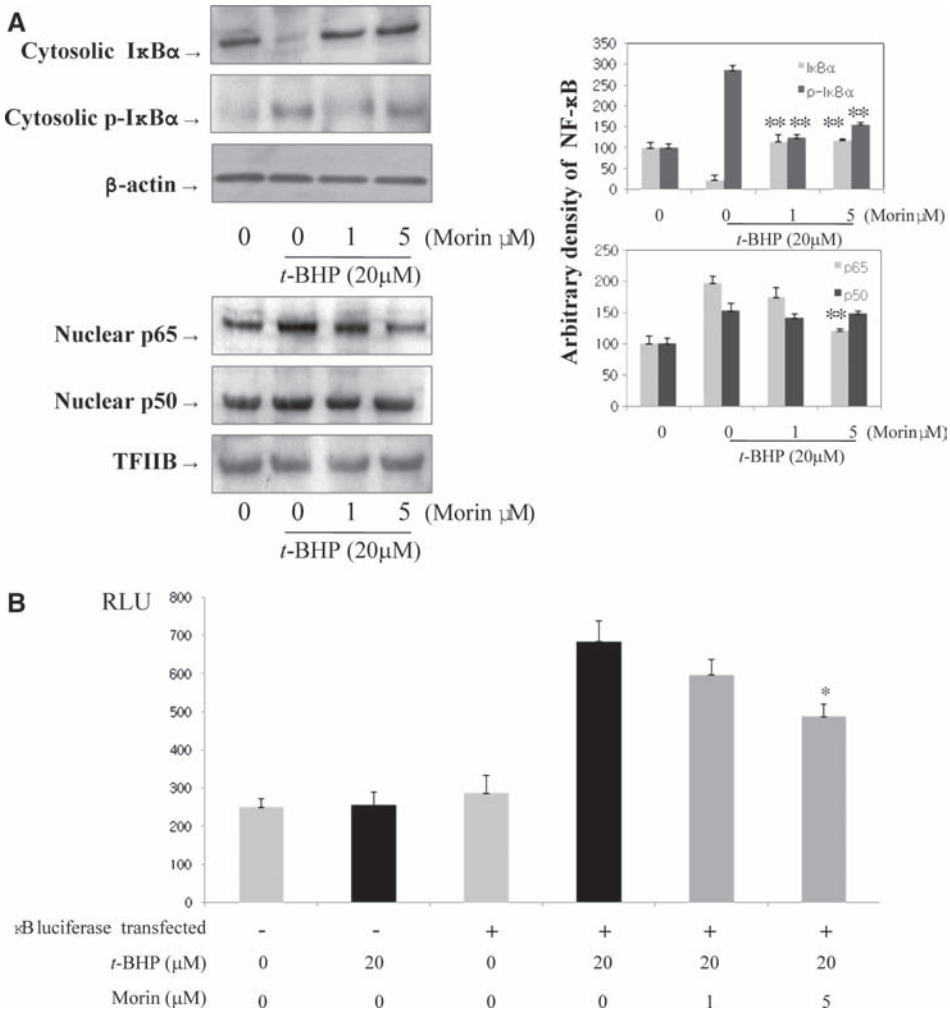

Figure 4. Effect of morin on *t*-BHP- induced NF-κB activation in YPEN-1 cells. (A) The cells were grown to 80% confluence in DMEM and changed serum free media. Pre-treatment with morin (1 or 5 μM) for 2 h and in the presence of 20 μM *t*-BHP for 1 h. Western blot was performed to detect p65 and p50 protein levels in the nuclear fraction. Levels were normalized to transcription factor IIB (TFIIB). IκBα and p- IκBα protein levels in cytosol fractions. Levels were normalized to β-actin. Values are the relative optical intensity of each band normalized as a percentage of the untreated control. $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs control, $*p < 0.05$, $**p < 0.01$ vs the group treated by only 20 μM *t*-BHP. (B) NF-κB activity was examined using a luciferase plasmid DNA, pTAL-NF-κB that contains a specific binding sequence for NF-κB. Luciferase assay was performed as described in the Materials and methods section. $^{\#\#}p < 0.001$ vs control, $*p < 0.05$ vs the group treated by only 20 μM *t*-BHP.

we examined the effect of morin on *t*-BHP-induced phosphorylation of the ERK, JNK and p38 proteins of MAPK in endothelial cells compared with PD98059 (ERK inhibitor), SP600125 (JNK inhibitor) and SB203590 (p38 inhibitor) (Figure 5). Western blot results indicated that *t*-BHP enhanced the phosphorylation; however, PD98059, SP600125 and morin pre-treatment (at 1 or 5 μM) inhibited *t*-BHP-induced phosphorylation of ERK and p38. However, interestingly SP600125 inhibited *t*-BHP-induced phosphorylation of JNK, but morin did not inhibit *t*-BHP-induced phosphorylation of JNK.

Reduction of t-BHP-induced NF-κB-dependent pro-inflammatory gene expression by morin

To determine the expression of *t*-BHP NF-κB dependent pro-inflammatory genes suppressed by morin, expressions of COX-2, 5-LOX and iNOS were examined by Western blot. These genes are known to be associated with inflammation, to have an NF-κB binding site in their promoter regions and to be controlled by NF-κB activation. Therefore, to elucidate the changes in NF-κB DNA-binding activity that correlates with NF-κB-dependent gene expression, we examined the expression of these genes. As shown in Figure 5, COX-2, 5-LOX and iNOS levels increased with *t*-BHP-induced RS, but morin decreased these levels. These results suggest that morin modulated NF-κB activation and NF-κB dependent gene expressions.

Discussion

Flavonoids are a class of secondary metabolites found abundantly in fruits and vegetables and are suggested

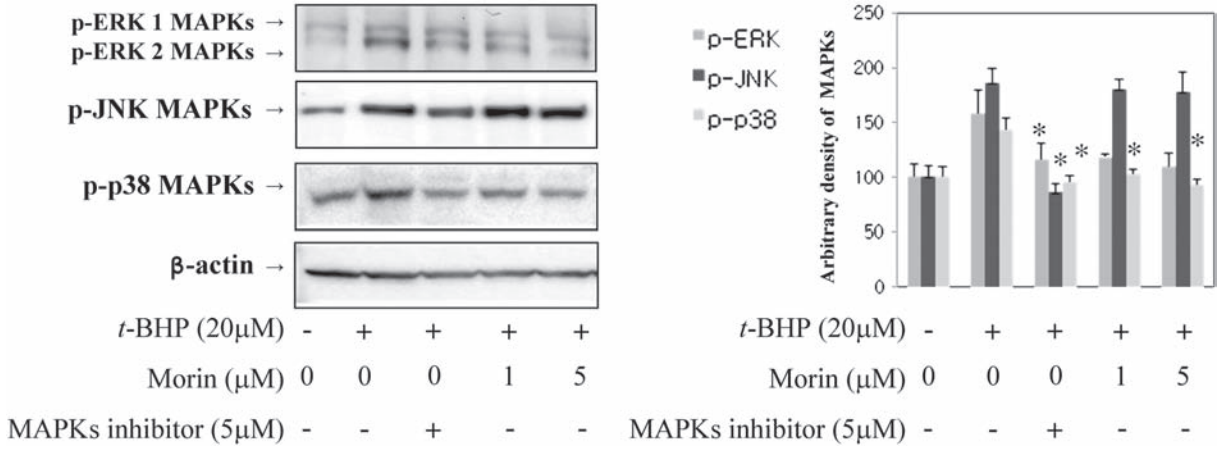

Figure 5. Effect of morin on *t*-BHP-induced phosphorylation of MAPKs in YPEN-1 cells. The cells were grown to 80% confluence in DMEM and changed to serum-free media. Pre-treatment with PD98059 (ERK inhibitor; 5 μM), SP600125 (JNK inhibitor; 5 μM), SB203590 (p38 inhibitor; 5 μM) and morin (1 or 5 μM) for 2 h and in the presence of 20 μM *t*-BHP for 30 min. Western blot was performed to detect p-ERK, p-p38 and p-JNK protein levels in cytosol fraction. Levels were normalized to *β*-actin. Values are the relative optical intensity of each band normalized as a percentage of the untreated control. $^{\#}p < 0.05$ vs control, $*p < 0.05$ vs the group treated by only 20 μM *t*-BHP.

to have several health benefits. The bioactivities of flavonoids are attributed to the presence of phenolic hydroxyl (OH) moieties on the structure. The association between the number of OH moieties and the antioxidant activity of flavonoid has been investigated extensively [26–28].

Morin, a member of the flavonoid family, has been reported to have cytoprotective properties against oxidative stress through its antioxidant ability. Previous papers report that morin exhibits antioxidant effects against free radicals activities, including scavenging superoxide and inhibiting xanthine oxidase activity [29,30]. Morin is also shown to have neuroprotective properties against cellular oxidative stress through its effective antioxidant effects [31]. Although previously morin's antioxidant effects on free radicals [32–34] and against oxidative stress [35,36] were reported, this report describes morin's modulation of the RS-induced NF-κB pathway.

In this current study, we investigated Morin's ability to directly scavenge SIN-1-generated RS *in vitro* and in YPEN-1 endothelial cells. SIN-1 is a RS donor, an excellent source of RS generation and is well-known to cause RS-induced damage. Our results show that morin is a strong anti-oxidant agent because it scavenged RS as effective as Trolox, a vitamin E analogue, that served as the positive control in our study. In subsequent experiments using endothelial cells, we were able to demonstrate that morin modulated the RS-induced NF-κB pathway through the MAPKs pathway by its RS scavenging activity.

Redox-sensitive transcription factor, NF-κB is a well known regulator of the expression of many genes important to the inflammatory response [38]. Several studies have reported that regulatory effects of morin on NF-κB activation. For instance, morin regulates the activation of NF-κB in hepatocellular carcinoma and primary neuron cultures cells of rat induced with diethylnitrosamine and glutamate [38,39], but its molecular mechanism is still unclear. Moreover, there are no of morin modulating the RS-induced NF-κB pathway by its RS scavenging activity. This is mportant because RS-induced NF-κB activation plays a key role in the pro-inflammatory response in endothelial cells and implicates vascular inflammation [40]. Under disturbed redox conditions, IkBa is phosphorylated at serine residues, ubiquitinated at lysine residues and degraded through the proteosomal pathway, thereby exposing the nuclear localization signals on the p50 and p65 heterodimer [41,42].

In this study, *t*-BHP induced RS activated NF-κB in the YPEN-1 cell system, but morin was found to inhibit the RS-induced NF-κB activation by suppressing IκBα phosphorylation and degradation and blunting the translocation of p65 and p50 sub-units. In addition, we determined that NF-κB binding activity was suppressed by morin. These inhibitory effects are highly likely due to a modulated ERK and p38 MAPKs pathway. Although morin inhibited phophorylation of ERK and p38, it had no response on JNK MAPKs. NF-κB activation led to the up-regulation of several major pro-inflammatory mediators such as COX-2, iNOS and adhesion molecules, VCAM-1 and ICAM-1. Morin down-regulated the expression of certain genes regulated by NF-κB, we postulate that morin modulates the activation of NF-κB and NF-κB-regulated gene expression induced by *t*-BHP-induced RS. Morin also suppressed NF-κB activation and down-regulated the expression of NF-κB-dependent pro-inflammatory genes.

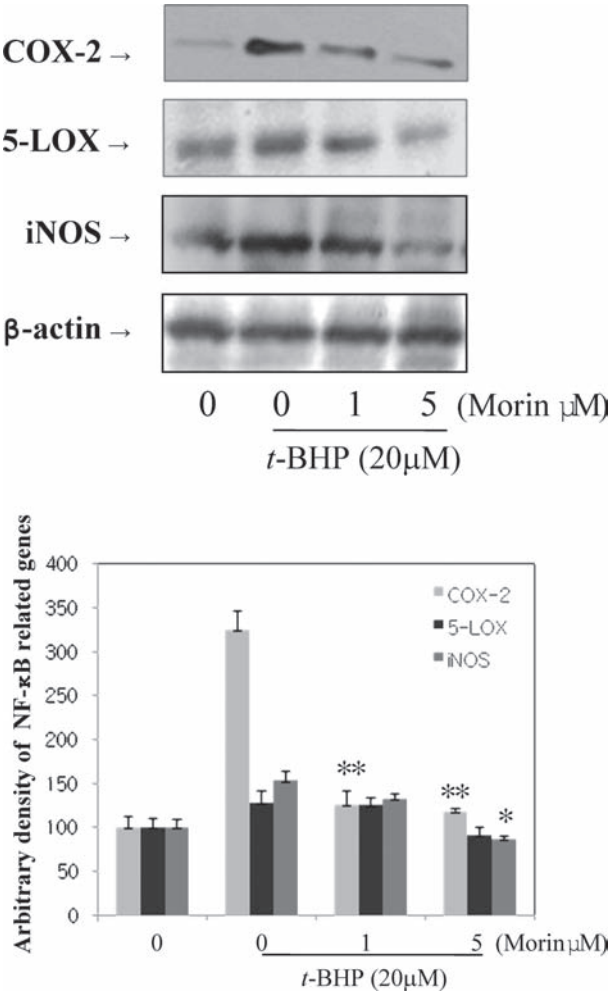

Figure 6. Effect of morin on *t*-BHP-induced NF-κB-dependant pro-inflammatory genes expression in YPEN-1 cells. The cells were grown to 80% confluence in DMEM and changed to serum-free media. Pre-treatment with morin (1 or 5 μM) for 2 h and in the presence of 20 μM *t*-BHP for 5 h. Western blot was performed to detect COX-2, 5-LOX and iNOS protein levels in cytosol fractions. Levels were normalized to β-actin. Values are the relative optical intensity of each band normalized as a percentage of the untreated control. $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs control, $^{*}p < 0.05$, $^{**}p < 0.01$ vs the group treated by only 20 μM *t*-BHP.

Putting these observations together, it seems clear that morin can scavenge RS, indicating its efficient anti-oxidative effect. Our current findings are among the first to reveal that in endothelial cells, morin modulates RS-induced NF-κB activation through the MAPKs pathway by way of its scavenging activity. In conclusion, we obtained evidence that morin's efficacious effect on the modulation of the NF-κB pathway and NF-κB dependant genes stems from its ability to scavenge RS, resulting in the suppression of pro-inflammatory genes. Based on these data, we propose that the anti-inflammatory and anti-oxidative properties of morin may have potential therapeutic applications in the treatment and prevention of RS-induced inflammatory processes and inflammatory-related diseases.

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