

Emodin protects against oxidative stress and apoptosis in HK-2 renal tubular epithelial cells after hypoxia/reoxygenation

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Abstract. The aim of the present study was to determine the effects of emodin, a natural compound with antioxidant properties, on oxidative stress and apoptosis induced by hypoxia/reoxygenation (H/R) in HK-2 human renal tubular cells. In HK-2 cells subjected to H/R, it was observed that pre-treatment with emodin lead to an increase in cellular viability and a reduction in the rate of apoptosis and the B-cell lymphoma 2 (Bcl-2)-associated X protein/Bcl-2 ratio. H/R alone caused a significant increase in the levels of reactive oxygen species and malondialdehyde ($P < 0.05$) and a significant decrease in the activities of superoxide dismutase, catalase and glutathione peroxidase ($P < 0.05$), relative to normoxic cells. In turn, parameters of oxidative stress were improved by emodin pre-treatment. In addition, emodin pre-treatment significantly inhibited the phosphorylation of extracellular signal-regulated protein kinase and c-Jun N-terminal kinase mitogen-activated protein kinases (MAPKs) induced by H/R ($P < 0.05$). These data suggest that emodin may prevent H/R-induced apoptosis in human renal tubular cells through the regulation of cellular oxidative stress, MAPK activation and restoration of the Bax/Bcl-2 ratio.

Introduction

Renal ischemia/reperfusion (I/R) injury typically occurs after renal transplantation and surgery, and is a leading cause of acute renal failure (1). Although reperfusion is a necessary approach to improve the survival probability of ischemic tissue, reperfusion itself can lead to additional cell injury, largely through the production of excessive reactive oxygen species (ROS) (2). ROS are a class of chemically reactive metabolites, including

superoxide anions, hydroxyl radicals and hydrogen peroxide. Overproduction of ROS directly triggers the oxidation of biological macromolecules, such as DNA, proteins and lipids, and modulates numerous signaling pathways, particularly those involving mitogen-activated protein kinases (MAPKs), which ultimately leads to cell death (3). MAPKs are a family of serine/threonine protein kinases that principally consists of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK). Previous results indicate that modulation of MAPK signaling pathways may have a role in ROS-induced cell apoptosis during I/R injury (4), suggesting that reducing the generation of ROS may be a useful therapeutic strategy in the prevention of I/R injury.

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone; Fig. 1A) is an active compound of various traditional Chinese herbs, including *Rheum officinale* and *Rheum palmatum* (5). Emodin has been demonstrated to have multiple biological activities, including anti-cancer, anti-diabetic, anti-inflammatory and antioxidant effects (6). It has also been documented that emodin protects against acute myocardial infarction through the inhibition of inflammation and apoptosis (7). In addition, emodin treatment may alleviate I/R injury in rat hearts (8). However, the effects of emodin on renal I/R injury are currently unknown.

In the present study, an *in vitro* model of renal I/R injury was established in human HK-2 renal tubular cells. HK-2 cells were treated with different concentrations of emodin prior to hypoxia/reoxygenation (H/R), and the viability and apoptotic rates of HK-2 cells were subsequently evaluated using MTT and annexin-V/propidium iodide (PI) staining assays, respectively. In addition, levels of intracellular ROS were measured using a 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) probe, and western blot analysis was performed to determine the levels of MAPK activation.

Materials and methods

Cell culture. HK-2, an immortalized proximal tubule epithelial cell line isolated from normal adult human kidney, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F12

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supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and were subcultured every 3-4 days after reaching 80% confluence.

H/R protocol and drug treatment. An HK-2 cell-based H/R model was established to simulate *in vivo* I/R injury, as described previously (9). To induce hypoxia, confluent HK-2 cells were incubated for 24 h in serum-free DMEM in a hypoxia chamber containing 95% N₂ and 5% CO₂ at 37°C. Following exposure to hypoxic conditions, cell medium was replaced with fresh oxygenated DMEM and cells were reoxygenated for 12 h in normoxic conditions (5% CO₂, 21% O₂ and 74% N₂) at 37°C.

To evaluate the effect of emodin on H/R injury, different concentrations (10-50 μM) of emodin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 0.1% dimethyl sulfoxide were added to cell cultures 2 h before the induction of H/R and incubated at 37°C. Prior to H/R experiments, the cytotoxicity of emodin against normoxic HK-2 cells was evaluated by exposing cells to different concentrations of emodin (10, 30, 50, 80 and 100 μM) in DMEM for 48 h at 37°C and measuring changes in cell viability. HK-2 cells cultured in DMEM under normoxic conditions were used as a vehicle control. Based on the results of the normoxic HK-2 cell viability assay, $\leq 50 \mu\text{M}$ emodin was used in all subsequent experiments. A negative control group (no H/R or emodin treatment) and positive control group (H/R-exposed cells lacking emodin treatment) were included.

Cell viability assay. Following the aforementioned treatments, HK-2 cells were seeded into 96-well plates at a density of 2,000 cells/well. Cells were then subjected to an MTT assay. In brief, 100 μl DMEM containing 10% FBS was replaced with 100 μl fresh DMEM containing 1 mg/ml MTT (Sigma-Aldrich; Merck KGaA). After 4 h of incubation at 37°C, media was replaced with 150 μl dimethyl sulfoxide to dissolve the formazan crystals. Absorbance was measured at 570 nm with a microplate reader (Model 3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis assay. The ratio of apoptotic cells was examined using an Annexin V-fluorescein isothiocyanate/PI kit (BD PharMingen, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, following the aforementioned drug treatments, cells were trypsinized, harvested and incubated in the dark at room temperature with Annexin V and PI for 15 min. Apoptotic cells (Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺) were analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using CellQuestPro 5.2 software (BD Biosciences).

Measurement of caspase-3 activity. As an indicator of apoptosis, the activity of caspase 3 enzyme was measured using a Caspase 3 Colorimetric Assay kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's protocol. Briefly, HK-2 cells were lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM dithiothreitol; Sigma-Aldrich; Merck KGaA) for 15 min, then centrifuged at 14,000 $\times g$ for 10 min at 4°C. Protein concentrations were quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce; Thermo

Fisher Scientific, Inc.). Cell lysates (200 μg) from each sample were used to determine caspase-3 activity. The assay was based on the release of chromophore molecules by the enzymatic cleavage of DEVD (Asp-Glu-Val-Asp, a caspase-specific peptide substrate conjugated to reporter ρ -nitroaniline molecules). Caspase 3 activity was determined by measuring absorbance of the released chromophore at 405 nm with a microplate reader (Model 3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. The protein content of HK-2 cells was isolated using a Total Cell Protein Extraction kit (EMD Millipore, Billerica, MA, USA). Protein concentrations were determined using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). An equivalent amount of protein (40 $\mu\text{g/lane}$) from each sample was separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Following blocking in 5% non-fat milk at room temperature for 1 h, membranes were incubated with primary antibodies (Table I) at 4°C overnight. Membranes were washed three times with Tris-buffered saline with 0.1% Tween-20 (5 min/wash) and incubated with secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; Table I) for 1 h at room temperature. Protein bands were developed with an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Inc.) and quantified by densitometric analysis using Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc.).

Measurement of ROS levels. Briefly, HK-2 cells in DMEM containing 10% FBS were incubated for 30 min at 37°C with 5 μM DCF-DA (Molecular Probes; Thermo Fisher Scientific, Inc.) and washed with phosphate-buffered saline (PBS). The fluorescence intensity of DCF was measured using a FACSCalibur flow cytometer (BD Biosciences).

Measurement of malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) activities. To evaluate the extent of oxidative stress in HK-2 cells following H/R and emodin treatment, the levels of MDA and the activities of SOD, CAT and Gpx in cells were measured using assay kits for MDA (A003-1), Total SOD (A001-1), CAT (A007-1) and Gpx (A005; all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively.

Statistical analysis. Data are expressed as mean \pm standard deviation. Each assay was performed in triplicate and was repeated three times. Statistical differences between groups were determined using one-way analysis of variance followed by a Tukey's post hoc test. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Emodin alleviates H/R-induced cell damage in HK-2 cells. The cytotoxicity of emodin (Fig. 1A) was evaluated in HK-2 cells using an MTT cell viability assay. As depicted in

Table I. Antibodies.

Primary antibody	Supplier	Dilution	Catalogue no.
Anti-Bcl-2	SCB	1:800	sc-7382
Anti-Bax	SCB	1:800	sc-7480
Anti-ERK	CST	1:300	4348
Anti-p-ERK	CST	1:300	4376
Anti-JNK	CST	1:300	9252
Anti-p-JNK	CST	1:300	9251
Anti-p38	CST	1:500	9212
Anti-p-p38	CST	1:500	9211
Anti-β-actin	SCB	1:1,000	sc-47778
Secondary antibody			
HRP-conjugated goat anti-mouse IgG	SCB	1:3,000	sc-2005
HRP-conjugated goat anti-rabbit IgG	SCB	1:3,000	sc-2004

CST, Cell Signaling Technology, Inc., (Danvers, MA, USA); SCB, Santa Cruz Biotechnology, Inc., (Dallas, TX, USA); Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; IgG, immunoglobulin G; JNK, c-Jun N-terminal kinase; p-, phosphorylated.

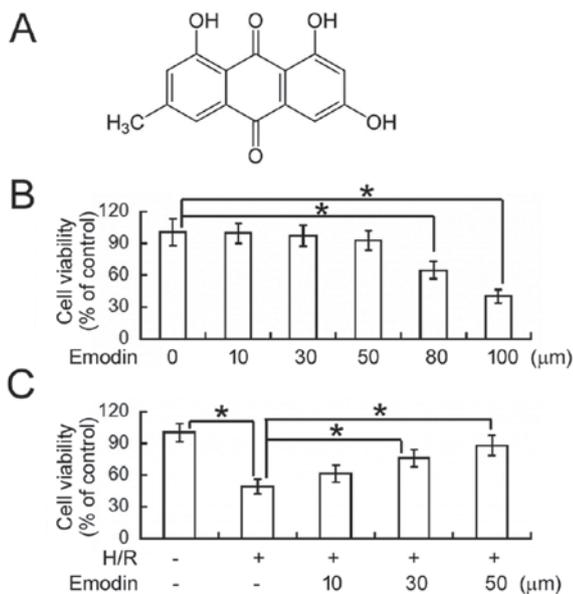


Figure 1. Emodin alleviates H/R-induced cellular damage in HK-2 cells. (A) Chemical structure of emodin. (B) Cytotoxicity of emodin in normoxic HK-2 cells was determined by an MTT assay following exposure to different concentrations of emodin for 48 h. (C) Measurements of the viability of H/R-exposed HK-2 cells with or without emodin pre-treatment. Data are representative of three independent experiments. *P<0.05. H/R, hypoxia/reoxygenation; HK-2, human renal tubular cell line.

Fig. 1B, exposure to $\leq 50 \mu\text{M}$ emodin for 48 h did not significantly affect the viability of normoxic HK-2 cells. However, emodin concentrations of 80 and 100 μM lead to ~35 and 60% reductions in cell viability, respectively, relative to vehicle-treated cells ($P<0.05$). Therefore, $\leq 50 \mu\text{M}$ emodin was used in all proceeding experiments unless otherwise stated.

Using an MTT assay, the effects of emodin on H/R-induced cellular injury were subsequently determined (Fig. 1C). It was observed that the viability of HK-2 cells was significantly

decreased following H/R exposure, relative to control cells under normoxic conditions ($P<0.05$). In turn, pre-treatment with 30 and 50 μM emodin significantly alleviated the inhibitory effects of H/R on cell viability ($P<0.05$) in a concentration-dependent manner.

Emodin inhibits HK-2 cell apoptosis following H/R. Annexin-V/PI staining and flow cytometry analysis were subsequently performed to determine whether emodin inhibited H/R-induced apoptosis in HK-2 cells (Fig. 2A). Following H/R exposure, an 8-fold increase in the percentage of Annexin V⁺ apoptotic cells was observed, indicating that the apoptotic rate of HK-2 cells was significantly increased, relative to normoxic controls ($P<0.05$). In turn, emodin pre-treatment (10 and 50 μM) significantly alleviated the pro-apoptotic effects of H/R, when compared to H/R treatment alone ($P<0.05$). To verify the effects of emodin on H/R-induced apoptosis, levels of caspase-3 activity were also measured, as an indicator of apoptotic rate. It was observed that HK-2 cells exposed to H/R had significantly higher (2.5-fold) levels of caspase-3 activity than normoxic cells ($P<0.05$; Fig. 2B). In turn, H/R-induced caspase-3 activation was significantly inhibited by pre-treatment with emodin (10 and 50 μM). In addition, levels of B-cell lymphoma (Bcl)-2 and Bcl-2-associated X protein (Bax), as primary regulators of apoptosis, were measured by western blot analysis. H/R-exposed cells exhibited a significant upregulation in Bax and a significant downregulation in Bcl-2, relative to normoxic cells (both $P<0.05$; Fig. 2C). In turn, pre-treatment with emodin significantly upregulated Bcl-2 and significantly downregulated Bax in H/R-exposed HK-2 cells, leading to restoration of the ratio between Bax and Bcl-2 (Fig. 2C).

Emodin alleviates oxidative stress in HK-2 cells following H/R. The effects of emodin on H/R-induced oxidative stress were evaluated. It was observed that levels of ROS and MDA were significantly increased following H/R treatment in HK-2 cells,

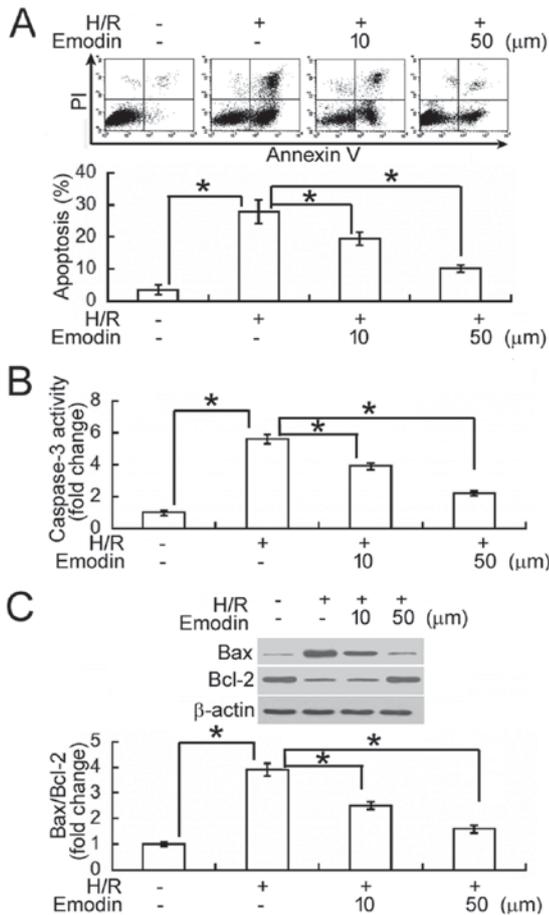


Figure 2. Emodin prevents H/R-induced apoptosis in HK-2 cells. HK-2 cells were pretreated with or without emodin 2 h before exposure to H/R and apoptotic changes were evaluated. (A) The rate of cell apoptosis was measured by flow cytometry following Annexin-V/PI staining. The lower and upper right quadrants show the proportions of early (Annexin V⁺/PI⁻) and late (Annexin V⁺/PI⁺) apoptotic cells, respectively. (B) Measurements of caspase-3 activity as an indicator of apoptosis. Results are expressed as the fold change in activity relative to normoxic cells. (C) Western blot analysis of Bax and Bcl-2 protein expression. Representative western blots are shown. Bax/Bcl-2 ratios were determined from immunoblotting data. Data are representative of three independent experiments. *P<0.05. H/R, hypoxia/reoxygenation; HK-2, human renal tubular cell line; PI, propidium iodide; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

relative to normoxic cells (P<0.05; Fig. 3A and B, respectively). In turn, pre-treatment with emodin (10 and 50 μM) significantly decreased the elevated levels of ROS and MDA induced by H/R (all P<0.05). By contrast, H/R treatment significantly decreased the activities of SOD, CAT, and GPx in HK-2 cells; an effect significantly reversed by emodin pre-treatment (P<0.05; Fig. 3C). These results suggest that emodin may aid to alleviate oxidative stress under H/R conditions.

Emodin inhibits H/R-induced activation of MAPK signaling.

The potential molecular mechanisms underlying the protective effects of emodin against H/R-induced cellular damage were investigated. As depicted in Fig. 4, levels of phosphorylated ERK and JNK MAPKs were significantly increased following exposure to H/R, relative to the normoxic control group (P<0.05). By contrast, the phosphorylation status of p38 MAPK was unaffected by H/R exposure. Notably, relative to the H/R group, emodin pre-treatment (10 and 50 μM)

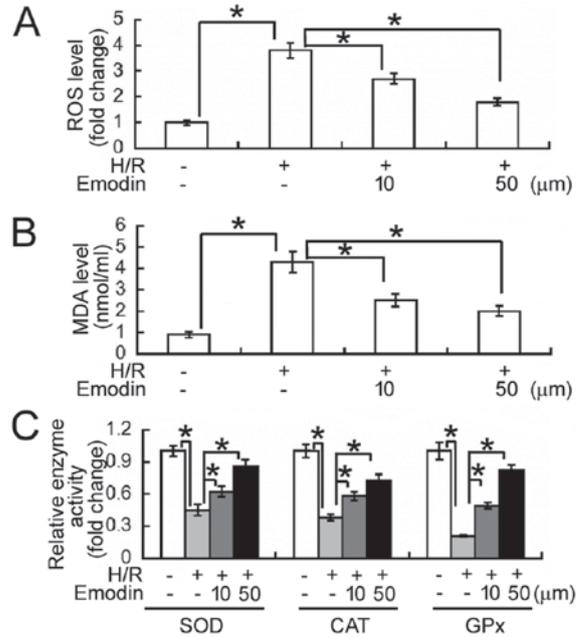


Figure 3. Emodin alleviates oxidative stress in HK-2 cells following H/R. Cells were pretreated with or without emodin 2 h before exposure to H/R and levels of oxidative stress were evaluated. The levels of (A) ROS and (B) MDA and the activities of (C) antioxidant enzymes (SOD, CAT and GPx) were measured. Data are representative of three independent experiments. *P<0.05. H/R, hypoxia/reoxygenation; HK-2, human renal tubular cell line; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

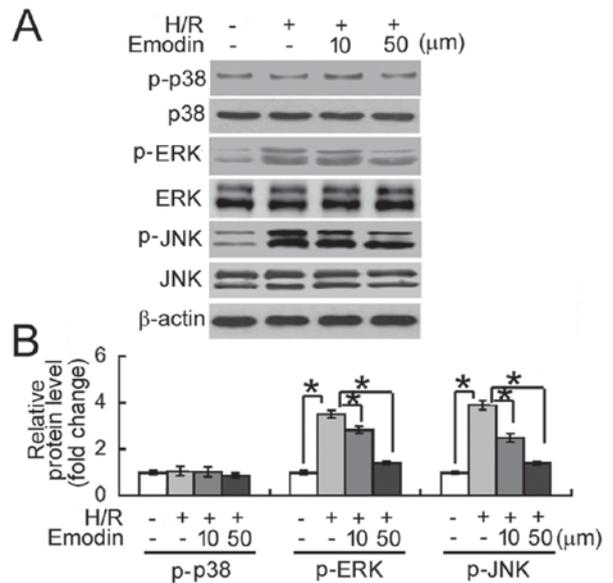


Figure 4. Emodin inhibits H/R-induced activation of MAPK signaling. HK-2 cells were pretreated with or without emodin 2 h before exposure to H/R and subjected to western blot analysis to determine the effects on MAPK signaling. (A) Representative western blots showing the expression and phosphorylation status of MAPKs (JNK, ERK and p38) are shown. (B) Densitometric analysis of p-ERK, p-JNK and p-p38 MAPKs. Data are representative of three independent experiments. *P<0.05. H/R, hypoxia/reoxygenation; HK-2, human renal tubular cell line; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

significantly inhibited H/R-induced phosphorylation of ERK and JNK MAPKs (all P<0.05).

Discussion

Oxidative stress mediated by ROS is involved in the pathogenesis of I/R injury (10). Numerous natural agents with antioxidant properties, including butin (11), catalpol (12) and ginsenoside Rg1 (13), have been evaluated as potential therapeutics in the prevention of ROS production during I/R. The natural compound emodin is of particular interest due to its multiple biological activities (6). Notably, emodin has demonstrated antioxidant activities in different pathological conditions (14,15). Xue *et al* (14) documented that emodin may protect against lung injury induced by cigarette smoke by suppressing the formation of ROS. In addition, Nemmar *et al* (15) demonstrated that emodin attenuated pulmonary inflammation and subsequent oxidative stress induced by diesel exhaust particles in mice. Therefore, the present study evaluated whether emodin had similar protective activities in renal I/R injury.

It was demonstrated that emodin pre-treatment significantly prevented reductions in the viability of HK-2 renal tubular cells following H/R. Furthermore, this protective activity of emodin was concentration-dependent. It has previously been demonstrated that renal tubular cells are sensitive to I/R injury and undergo substantial levels of cell apoptosis (16). In the present HK-2 cell model, H/R exposure significantly promoted cell apoptosis, as determined by Annexin-V/PI staining and measurements of caspase-3 activity. Notably, the H/R-induced apoptotic response in HK-2 cells was significantly prevented by emodin pre-treatment. Bcl-2 is an antiapoptotic protein that maintains the integrity of the outer mitochondrial membrane, and is inhibited by other members of the Bcl-2 family (including Bax), leading to cytochrome *c* release and caspase-3 activation (17). It has previously been documented that Bcl-2 protects tubular epithelial cells from I/R injury through the inhibition of apoptosis (16). In addition, it has been demonstrated that adenoviral delivery of Bcl-2 may inhibit proximal and distal tubular apoptosis induced by renal I/R (14). Consistent with these previous results, the present study observed that the protective activity of emodin in H/R-exposed HK-2 cells was associated with increased Bcl-2 and decreased Bax expression. Therefore, emodin may aid the protection of renal tubular cells against H/R-induced apoptosis by restoring the ratio between Bax and Bcl-2.

The present study also aimed to determine whether the protective effects of emodin were associated with the modulation of ROS generation and MAPK signaling. It was observed that H/R-induced oxidative stress in HK-2 cells was significantly alleviated by emodin treatment, as indicated by lower levels of ROS and MDA. Furthermore, the activities of antioxidant enzymes (SOD, CAT and Gpx) in H/R-exposed cells were significantly increased by emodin. The results suggest that emodin exerts antioxidant effects in renal tubular cells under H/R conditions. It has previously been suggested that oxidative damage induced by ROS overproduction may be in part mediated by alterations in MAPK activation (3). Bae *et al* (18) also documented that 4-hydroxy-2-hexenal, a lipid oxidation-derived toxic product, induced ROS production and apoptosis in HK-2 cells through the activation of ERK and JNK MAPKs, and that pharmacological inhibition of ERK or JNK attenuated the damaging effects of 4-hydroxy-2-hexenal.

Similarly, the present study found that emodin pre-treatment suppressed the activation of ERK and JNK MAPKs in H/R-exposed HK-2 cells. Inactivation of MAPK signaling by emodin has also been documented in several other biological conditions (19,20). For instance, in a rat model of sepsis, emodin attenuated lung injury through inhibition of p38 MAPK (20). The regulation of ROS production and MAPK activation may represent an important mechanism for the protection against H/R-induced cellular injury induced by emodin.

In conclusion, the present data indicates that emodin prevents H/R-induced apoptosis in human renal tubular cells. In addition, the protective effects of emodin were associated with the regulation of cellular oxidative stress and MAPK activation, along with the restoration of the Bcl-2/Bax ratio. Further studies are now required in animal models to validate the protective effects of emodin against renal I/R injury.

Acknowledgements

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