Mangiferin inhibits hypoxia/reoxygenation-induced alveolar epithelial cell injury via the SIRT1/AMPK signaling pathway

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Abstract. Lung ischemia-reperfusion injury (LIRI) is one of the complications that can occur after lung transplantation and may lead to morbidity and mortality. Mangiferin (MAF) is a naturally occurring glucosyl xanthone that has been documented to possess anti-inflammatory, immunomodulatory and potent antioxidant effects. The purpose of the present study was to investigate the effect of MAF on LIRI using a hypoxia-reoxygenation (H/R) cell model. In the present study, the viability of lung alveolar epithelial cells (A549) and H/R-A549 were detected by MTT assay. ELISA was used to evaluate the expression levels of IL-6 and IL-1β. TUNEL assay and western blotting were used to evaluate the apoptosis. In addition, H/R-A549 cells were treated with sirtinol, which is known inhibitor of sirtuin 1 (SIRT1) activity, to determine the effects of MAF on proteins associated with the SIRT1/5'AMP-activate protein kinase (AMPK) signaling pathway using western blotting. The results showed that 20 µM MAF exerted a protective effect on A549 cells against H/R mediating no clear cytotoxic effects. In terms of inflammation, MAF reduced IL-6, IL-1β, cyclooxygenase-2 and inducible nitric oxide synthase expression, which was accompanied by activation of the SIRT1/AMPK signaling pathway. In addition, compared with those in the group treated with sirtinol, expression of SIRT1, Bcl-2 and AMPK activity were elevated in MAF-treated H/R-A549 cells, whereas the expression of Bax, cleaved caspase-3 and cleaved caspase-9 was suppressed. TUNEL analysis of H/R-A549 cells treated with MAF in combination with sirtinol revealed that treatment with sirtinol blocked the SIRT1/AMPK signaling pathway and increased the apoptosis rate compared with the MAF group. Taken together, results of the present study revealed that MAF could inhibit lung H/R cell injury through the SIRT1/AMPK signaling pathway.

Introduction

Lung ischemia-reperfusion injury (LIRI) is one of the complications that can potentially arise after lung transplantation (1). Following lung transplantation, organ ischemia and subsequent reperfusion is unavoidable and frequently leads to acute sterile inflammation in a process known as ischemia reperfusion injury (1,2). LIRI may also develop into pulmonary infection, acute lung injury and bronchiolitis obliterans syndrome, thereby reducing the post-transplant survival rate and increasing risk of mortality (3).

Sirtuins (SIRT) belong to a protein family that is responsible for regulating various intracellular events, including cell proliferation, apoptosis and cell migration (4,5). In particular, sirtuin 1 (SIRT1) is one of the most studied isoform of the sirtuin family (6,7). SIRT1 is also known as a NAD+‑dependent histone deacetylase and has been revealed to be an important modulator of energy metabolism (6,7). Notably, it has been previously shown that LIRI can be alleviated by activating SIRT (8-10).

5'AMP-activated protein kinase (AMPK) is an ubiquitous energy sensor enzyme and is considered to be a downstream effector of SIRT1, which serves a key role in the regulation of energy homeostasis and cell survival (4,11,12). AMPK has been reported to exert SIRT1-dependent anti-inflammatory activities in sepsis-induced acute lung injury (13).

Over the past decade, there has been growing interest in the potential application of natural bioactive components isolated from plants for therapeutic uses. Mangiferin (MAF) is a C-glucosyl xanthone that is present at high levels in higher plants such as mango (Mangifera indica L.) (14,15). It has been demonstrated to possess numerous pharmacological activities, including antiviral, anticancer, antioxidative, antiaging, immunomodulatory and analgesic effects (15-18). Furthermore, it has been reported that MAF can prevent liver lipid metabolism disorders by regulating the SIRT1/AMPK pathway (19) whilst also inhibiting pulmonary fibrosis (20). However, the effects of MAF on LIRI remains poorly understood. Therefore, the aim of the present study was to evaluate the effects of MAF on LIRI using an in vitro hypoxia/reoxygenation (H/R) cell model and explore its possible mechanism.
Materials and methods

Cell culture and modeling. The human alveolar epithelial cells (A549) were obtained from American Type Culture Collection (ATCC). They were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from Beijing Solarbio Science & Technology Co., Ltd.). 100 U/ml penicillin and 100 pg/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C.

A H/R-A549 cell model was established. Briefly, A549 cells were exposed to anoxia for 24 h (1% O₂, 5% CO₂ and 94% N₂) at 37°C followed by 4 h of reoxygenation (5% CO₂ and 95% air) at 37°C (H/R group) (21,22).

The cell model with the SIRT1/AMPK signaling pathway blocked (H/R-A549 + sirtinol) was established. Sirtinol stock solution (10 mM) (Sigma-Aldrich; Merck KGaA) and MAF (Sigma-Aldrich; Merck KGaA; Fig. 1A) stock solution (50 mM) were prepared in dimethyl sulfoxide and stored at -80°C until further use. H/R-A549 cells were treated with 15 µM of sirtinol for 72 h at 37°C (23). Subsequently, the cells were cultured for another 24 h with 20 µM MAF at 37°C (H/R + MAF + sirtinol group). Additionally, H/R-A549 cells were incubated with 20 µM MAF at 37°C for 24 h (H/R + MAF group).

Cell viability test. Cell viability was measured using MTT assay. A549 and H/R-A549 cells (5x10⁴/well) were each incubated in 96-well plates with various concentrations of MAF (5, 10, 20 and 50 µM). Using the RPMI-1640 medium to dilute the drug stock solution, the cell toxicity of DMSO was negligible due to the use of ≥1,000-fold dilution. After incubation for 24 h, 20 µl MTT (5 mg/ml) was added to each well and cells were incubated for a further 4 h at 37°C. A buffer solution (10% SDS, 5% isopropanol and 0.1% HCl) was then added to solubilize the MTT formazan crystals overnight. The absorbance in each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Untreated cells were used as a control.

ELISA cytokine assay. A549 and H/R-A549 cells (5x10⁴/well) were inoculated into 96-well plates and treated with different MAF concentrations (5, 10, 20 and 50 µM) for 24 h at 37°C. Subsequently, the cell culture medium was centrifuged at 2,000 x g for 4 min at 4˚C to remove debris and the supernatant was collected for assay. The levels of IL-6, IL-1β in cells were quantified using their corresponding ELISA assay. A549 and H/R-A549 cells (5x10³/well) were each incubated with 20 µM MAF at 37°C for 24 h, 20 µl MTT (5 mg/ml) was added to each well and cells were incubated with 50 µg/ml DAPI at 37°C in the dark for 5 min. An anti-fade solution (Beijing Solarbio Science & Technology Co., Ltd.) was dropped onto the area containing the treated cells and the sections were mounted onto the slides. Finally, all samples were imaged in three random fields per coverslip using a fluorescence microscope (magnification, x100) to view the green fluorescence at 520±20 nm and blue DAPI at 460 nm.

Western blotting assay. The protein samples from the cells were extracted using the cell lysis buffer (cat. no. P0013; Beyotime Biotechnology Inc.) and the protein content was measured using a BCA Protein Assay Kit (cat. no. P0012S; Beyotime Biotechnology Inc.). The protein samples (40 µg) were subjected to 15% SDS-PAGE for separation and transferred onto nitrocellulose membranes. After blocking in fresh 5% non-fat milk at room temperature for 2 h, the membranes were incubated overnight at 4°C with primary antibodies, followed by incubation with a HRP-conjugated secondary antibody at room temperature for 2 h. An enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Inc.) was used to visualize the signals. The antibodies (Affinity Biosciences) used included: Anti-SIRT1 (cat. no. DF6033; 1:1,000), anti-phosphorylated (p–)-AMPK (cat. no. AF3423; 1:1,000), anti-AMPK (cat. no. AF6423; 1:1,000), anti-Cox-2 (cat. no. AF7003; 1:1,000), anti-iNOS (cat. no. AF0199; 1:1,000), anti-Bcl-2 (cat. no. AF6139; 1:1,000), anti-Bax (cat. no. AF0120; 1:1,000), anti-cleaved-caspase-3 (cat. no. AF7022; 1:1,000), anti-cleaved-caspase-9 (cat. no. AF5240; 1:1,000), anti-GAPDH (cat. no. AF7021; 1:5,000) and goat anti-rabbit IgG secondary antibody (cat. no. S0001; 1:5,000). Protein expression levels were semi-quantified using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc.).

Statistical analyses. All data were expressed as the mean ± standard deviation. statistical analysis was performed with GraphPad Prism 8.0 software (GraphPad Software, Inc.). Differences between the means of the groups were compared using a one-way ANOVA followed by Tukey's test. Each experiment was repeated ≥ three times. P<0.05 was considered to indicate a statistically significant difference.

Results

MAF promotes the viability of H/R-A549 cells. As shown in Fig. 1B, MAF at concentrations of <50 µM, could not exert toxicity on A549 cells. However. 50 µM MAF was found to be significantly toxic to cells. According to Fig. IC, cell viability after H/R induction was significantly decreased compared with that in the control group. By contrast, the viability of H/R-A549 cells was increased significantly after treatment with 20 µM MAF. However, after treatment with 50 µM MAF, cell viability was markedly decreased compared with that in the 20 µM MAF treatment group. Therefore, in the subsequent experiments, the maximum concentration of MAF used was 20 µM.

MAF inhibits inflammation in H/R-A549 cells. The levels of the inflammatory markers in H/R-A549 cells treated with...
different concentrations of MAF were measured. As shown in Fig. 2A-C, ELISA analysis showed that compared with those in the control group, H/R significantly upregulated the expression levels of IL-6 and IL-1β, whilst significantly downregulating the expression level of IL-10. By contrast, the H/R-induced upregulation of IL-6 and IL-1β and downregulation of IL-10 were significantly reversed in the MAF-treated groups in a dose-dependent manner. In addition, the protein expression levels of Cox-2 and iNOS were also significantly increased in the H/R induced group compared with those in the control group, whilst those in the MAF-treated groups were significantly reversed compared with those in the H/R group. Taken together, these data suggest that MAF treatment can inhibit the H/R induced inflammatory response.

**MAF inhibits apoptosis in H/R-A549 cells.** Nuclear DNA fragmentation is an important biochemical event during apoptosis in many cell types and was therefore measured by TUNEL assay in the present study. The TUNEL results showed that A549 cell apoptosis was markedly increased after H/R induction (Fig. 3A). By contrast, after the addition of MAF, the H/R-induced cell apoptosis was reduced (Fig. 3A). In addition, compared with those in the control group, the expression levels of Bax, cleaved-caspase-3 and cleaved-caspase 9 protein were significantly increased in the H/R group, whilst those of Bcl-2 were reduced (Fig. 3B). Notably, the expression level of Bcl-2 was significantly increased by 10 and 20 µM MAF treatment compared with that in the H/R group (Fig. 3B). In addition, the expression levels of Bax, cleaved-caspase-3 and cleaved-caspase-9 were significantly decreased in 20 µM MAF treatment group compared with those in the H/R group (Fig. 3B). These results suggest that MAF can inhibit H/R-induced apoptosis in A549 cells.

**MAF activates the SIRT1/AMPK signaling pathway.** To verify the hypothesis that MAF may serve a role in the SIRT1/AMPK signaling pathway, the protein levels of SIRT1 and ratio of p-AMPK/AMPK were evaluated in H/R cells by western blotting assay. As shown in Fig. 4, compared with those in the control group, the protein expression of
SIRT1 and ratio of p-AMPK/AMPK were significantly decreased in the H/R-induced group. By contrast, compared with the H/R-induced group, the protein levels of SIRT1 and ratio p-AMPK/AMPK were significantly increased in the 10 and 20 µM MAF treatment groups (Fig. 4). Since a concentration of 20 µM MAF resulted in a highly significant increase in SIRT1 and p-AMPK/AMPK levels, 20 µM MAF was used subsequently to explore the effects of MAF on the SIRT1/AMPK signaling pathway.

MAF inhibits H/R injury in cells through the SIRT1/AMPK signaling pathway. To understand whether the SIRT1/AMPK signaling pathway affected H/R-induced inflammation and apoptosis, the SIRT1 inhibitor sirtinol was used to intercept the SIRT1/AMPK signaling pathway following MAF treatment. As shown in Fig. 5A-D, after blocking the SIRT1/AMPK signaling pathway, the protective effects of 20 µM MAF against inflammation were reversed. Compared with those in the MAF treatment group (H/R + MAF), the pathway inhibition group (H/R + MAF + sirtinol) significantly upregulated the expression levels of IL-6 and IL-1β, whilst significantly downregulating the expression level of IL-10 (Fig. 5A-C). In addition, the protein expression levels of Cox-2 and iNOS were also significantly increased in the H/R + MAF + sirtinol group compared with those in the H/R + MAF group (Fig. 5D). After inhibiting the SIRT1/AMPK signaling pathway, the rate of apoptosis in MAF treatment was markedly increased compared with that in the H/R + MAF group (Fig. 5E). Compared with
Figure 4. Western blot analysis and quantification of SIRT1 expression and AMPK activation in alveolar epithelial cells and H/R cells after treatment with different concentrations of MAF. ***P<0.001 vs. control; *P<0.05 and ###P<0.001 vs. H/R. MAF, mangiferin; H/R, hypoxia-reoxygenation; SIRT1, sirtuin 1; AMPK, 5’AMP-activated protein kinase.

Figure 5. Effect of MAF and sirtinol on H/R-induced cell injury and the status of the sirtuin 1/5’AMP-activated protein kinase signaling pathway. The levels of (A) IL-6, (B) IL-1β and (C) IL-10 in H/R-A549 cells were detected by ELISA. (D) Western blot analysis of Cox-2 and iNOS protein expression in H/R-A549 cells. (E) Representative TUNEL images of alveolar epithelial cells. Scale bars, 250 µm. (F) Western blot analysis and (G) statistical quantification of Bcl-2, Bax, cleaved-caspase-3 and cleaved-caspase-9. ***P<0.001 vs. Control. ###P<0.001 vs. H/R. &P<0.05, &&P<0.01 and &&&P<0.001 vs. H/R + MAF. MAF, mangiferin; H/R, hypoxia-reoxygenation; Cox-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase.
those in the MAF treatment group, the protein expression levels of Bax, cleaved-caspase-3 and cleaved-caspase-9 were significantly increased after the addition of sirtinol, whereas Bcl-2 expression was significantly reduced (Fig. 5F). Altogether, these results suggest that MAF can activate the SIRT1/AMPK signaling pathway to inhibit H/R-induced A549 cell injury.

Discussion

The success of lung transplantation was limited by the high rates of primary graft dysfunction due to ischemia-reperfusion injury, which is characterized by potent inflammation, alveolar damage and vascular permeability (1,24). Ischemia-reperfusion injury that occurs following lung transplantation typically activates the innate immune system to induce inflammation (1,9). In turn, this inflammation can enhance acute allograft rejection, impair transplant tolerance and accelerate the progression of chronic rejection (25). A number of reports in cellular and animal models have shown that the levels of inflammatory factors, including IL-6 and IL-1β, were significantly increased after lung injury, whereas those of immunosuppressive factors, including IL-10, were decreased (26,27). Therefore, it is necessary to reduce the occurrence of inflammation to mitigate the damage induced by LIRI.

In recent years, the potential application of naturally occurring components of plants for clinical uses has attracted much attention (28-30). MAF is a natural constituent of foods and traditional herbal medicines, such as Mangifera indica, Anemarrhena asphodeloides and Coffea pseudozanguebariae, that exhibits almost no adverse effects or toxicity (31). In addition, it possesses numerous pharmacological activities, such as anti-inflammatory, immunomodulatory and antioxidative effects (15). In the present study, MAF exerted little to no toxicity on A549 cells but mediated protective effects on cells after H/R injury, which was similar to previous reports that MAF attenuated myocardial ischemia-reperfusion injury and lung injury (32,33). When the concentration of MAF used was <20 µM, H/R injury was protected by MAF in a dose-dependent manner.

A previous study showed that MAF could improve cell viability after H/R injury, which clarified the protective effects of MAF against myocardial injury (32). In addition, on inflammation, Wang et al found that MAF pretreatment significantly inhibits ischemia-reperfusion-induced elevated expression levels of TNF-α and IL-1β (34). Similarly, in the present study, the increased levels of IL-6 and IL-1β induced by H/R were markedly inhibited after MAF pretreatment. Previous studies have demonstrated that MAF can suppress apoptosis by reducing the protein expression levels of cleaved-caspase-3, caspase-9 and Bax whilst increasing those of Bcl-2 (35-37). Likewise, the apoptosis data found in the present study showed that MAF downregulated the expression levels of Bax, cleaved-caspase-3 and cleaved-caspase-9 protein and increased the expression of Bcl-2 to reduce apoptosis, consistent with the previously reported data aforementioned. Therefore, based on these data, the present study suggests that MAF exerted anti-inflammatory and anti-apoptotic effects on A549 cells.

Mechanistically, MAF exerts anti-inflammatory effects by activating the SIRT1 signaling pathway (38,39). Various studies have revealed that SIRT1 is a key component in a number of stress-related pathways, including cell apoptosis, cellular senescence and angiogenesis (40,41). In addition, SIRT1 has been proposed to be an attractive therapeutic target for myocardial ischemia-reperfusion injury (42,43). SIRT1 activation can inhibit the cardiac inflammatory response following ischemia-reperfusion (44). Once activated, SIRT1 can mediate a wide range of downstream signaling pathways, such as AMPK, peroxisome proliferator-activated receptor γ coactivator 1α and endothelial nitric oxide synthase (39,42,45). In the present study, the protein expression of SIRT1 and AMPK was increased in a dose-dependent manner after MAF treatment. After the addition of sirtinol, a SIRT1 inhibitor, the anti-inflammatory and anti-apoptotic effects of MAF were reversed. Therefore, MAF can activate the SIRT1/AMPK signaling pathway. Taken together, activation of the SIRT1/AMPK signaling pathway confer protective effects against ischemia-reperfusion injury.

In the present study, the effects of MAF were evaluated on an in vitro A549 cell model of LIRI using a cellular H/R model. The results showed that MAF could induce the activation of the SIRT1/AMPK signaling pathway, where it protected A549 cell injury induced by H/R by inhibiting inflammation and apoptosis. Application of MAF therefore provides a promising strategy for the treatment of LIRI. However, the fact that the present study only used in vitro and not in vivo models and examined the effects of MAF concentration on inflammation and apoptosis are limitations of the present study. In addition, further elucidation in clinical samples is required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

XC and JH conceived and designed the study, acquired and interpreted the data and revised it for critically important intellectual content. XC and JH confirm the authenticity of all raw data in the present study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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