Kaempferol Regulates miR-15b/Bcl-2/TLR4 to Alleviate OGD-Induced Injury in H9c2 Cells

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Summary
Ischemic heart disease (IHD) is one of the world’s leading causes of human death. Kaempferol (Kae) was proved to have anti-inflammatory, antioxidant, and anticancer effects. Such properties suggested that it might play protective roles in IHD. In this study, we have attempted to disclose the potential regulating mechanisms of Kae in primary cardiomyocytes and H9c2 cells.

Cells were first stimulated by oxygen-glucose deprivation (OGD) and then exposed to Kae. CCK-8 assay and flow cytometry were used to examine cell characteristics. Quantitative reverse-transcription polymerase chain reaction was utilized to test the expression levels of miR-15b and TLR4. Afterward, cell transfection, dual-luciferase activity assay, and western blot were used to explore the potential mechanisms.

OGD treatment suppressed cell viability, whereas it enhanced cell apoptosis. Besides, OGD treatment enhanced the expression of apoptosis-associated proteins. Kae exposure, however, attenuated the effects that OGD-induced. Further experiments showed that Kae exposure promoted down-regulation of miR-15b, Bcl-2 and TLR4 were a target of miR-15b. Moreover, Kae enhanced the expression of key factors involved in PI3K/AKT and Wnt/β-catenin pathways, whereas miR-15b mimic reversed the Kae-triggered effects.

This investigation revealed that Kae diminished OGD-triggered cell damage through down-regulating miR-15b expression via activating PI3K/AKT and Wnt3a/β-catenin pathways. (Int Heart J 2020; 61: 585-594)

Key words: Ischemic heart disease, Oxygen glucose deprivation, Cell apoptosis, Wnt/β-catenin and PI3K/AKT pathways

Globally, ischemic heart disease (IHD) is one of the leading causes of human death.1,2 The main reason of IHD is the decreased blood supply from coronary arteries to the heart, resulting in insufficient oxygen and energy delivered to the myocardium.3 These conditions result in myocardial tissue hypoxia, cell apoptosis and necrosis, and even myocardium dysfunction in severe cases.4 It is also reported that IHD accounts for 10.3% of patients who were readmitted within 30 days after coronary artery bypass surgery.5 Moreover, the pathological process of IHD is generally believed to be closely related to ischemia/reperfusion (I/R) injury,6 and accumulating studies have reported the strategies developed for reducing I/R injury.7,8

Kaempferol (Kae), one of the flavonoids, is widely distributed in the nature. In earlier studies, it had been proven to have anti-inflammatory,9 antioxidant10 and anti-cancer11 effects. These properties it possesses indicated that it might play a protective role in IHD. A previous research performed by Chen, et al. verified that Kae diminished hyperglycemia-evoked injuries in diabetic cardiomyopathy through repressing oxidative stress and inflammatory.12 Moreover, Guo, et al. illustrated that Kae took part in the protection of cardiomyocytes from the injuries induced by anoxia/reoxygenation treatment through mitochondrial pathway.13 Furthermore, Kim, et al. revealed that Kae successfully protected the cardiac damage evoked by I/R via regulating endoplasmic reticulum stress.14 However, the mechanism of Kae regulation of I/R-induced injury still needs further study.

Several previous studies stated that microRNAs (miRNAs) are involved in the protection of myocardial cells against cell injuries induced by I/R or hypoxia/re-oxygenation.15-21 miR-15b is a member of miR-15 family. Most of the investigations related to cardiac-related diseases involving miR-15b are focused on the effects of I/R treatment on miR-15b expression and its regulation effects on cell apoptosis. For example, an earlier study conducted by Liu, et al. clarified that the expression of miR-15b was up-regulated by I/R treatment.22 Besides, Hullinger, et al. revealed that the expression of miR-15b in infarcted region of porcine cardiac samples was notably increased.23 Other researches elucidated that miR-15b aggravated apoptosis of hypoxia/reoxygenation-evoked myocardial...
with 95% O2 and 5% CO2 at 37°C for 24 hours to induce OGD injury. Afterward, the culture medium was discarded and a glucose-free medium containing 4 mM L-glutamic acid (BBI Solution, Shanghai, China), 100 μg/mL streptomycin (Sangon Biotech, Shanghai, China), 100 μg/mL streptomycin (Sangon Biotech, Shanghai, China), and 20%/10% fetal bovine serum (FBS, Gibco-BRL, Carlsbad, CA, USA) and were cultivated in an incubator containing 5% CO2 at 37°C for 4 hours to induce OGD injury. Afterward, the culture medium was substituted with DMEM containing glucose and FBS, and the cells were maintained in an incubator filled with a mixture of 95% N2 and 5% CO2 and was maintained at 37°C for 4°C, and the supernatant was discarded. Afterward, cells were suspended with 200 μL of Binding Buffer (Sigma, St. Louis, Missouri, USA). Then, 10 μL of Annexin V-FITC and 5 μL of PI were added, and the mixture was subsequently reacted for 15 minutes in dark condition. Finally, the apoptotic cells were detected by using flow cytometer (Becton, Dickinson and Company, New Jersey, USA).

**Cell transfection:** The sequences of miR-15b mimic, negative control (NC) mimic, miR-15b inhibitor (in) and NC in were synthesized by Sangon Biotech (Shanghai, China). The non-treated and OGD + Kae-treated primary cardiomyocytes and H9c2 cells were respectively transfected with these sequences. Cells were collected at 48 hours post-transfection in the following trails.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR):** Total RNA from untreated cells, OGD-induced, and OGD + Kae treated cells as well as miR-15b mimic, NC mimic, miR-15b in, and NC in transfected cells was extracted by using Trizol reagent (TAKARA, Beijing, China) and then treated with DNaseI (Ambion, Thermo, Massachusetts, USA). The concentration and purity of total RNA were respectively detected by using Nanodrop 3000 (Thermo Scientific, Massachusetts, USA) and agarose gel electrophoresis. The cDNA was synthesized by using reverse transcription kit (TAKARA, Beijing, China). qRT-PCR was conducted by using SYBR® Premix EX Taq™ (Perfect Real time) (TAKARA, Beijing, China), and β-actin was used as an internal reference gene. Data were calculated by using 2−ΔΔCT.

**Western blot:** Total protein was taken out of the collected cells by using RIPA Lysis Buffer I (Sangon Biotech, Shanghai, China), and the content of extracted protein was measured by BCA method (Solarbio, Beijing, China). Afterward, the proteins were first separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then the blots were transferred on to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% bovine serum albumin (BSA, BBI Solution, Crumlin, UK) at 25°C for 1 hour, the primary antibodies directly against Pro-caspase-3 (ab13847, abcam), Cleaved-caspase-3 (ab9822, abcam), Pro-caspase-8 (ab10833, abcam), Cleaved-caspase-8 (ab25901, abcam), Pro-caspase-9 (C 7729, Millipore Sigma), Cleaved-caspase-9 (SAB4502569, Millipore Sigma), Bcl-2 (ab196495, abcam), Toll-like receptor 4 (TLR4) (ab217274, abcam), t-PI3K (ab151549, abcam), p-PI3K (ab182651, abcam), t-AKT (ab8805, abcam), p-AKT (ab38449, abcam), Wnt3a (ab219412, abcam), β-catenin (ab16051, abcam) and β-actin (ab8227, abcam) were added and maintained at 4°C overnight. The next day, the membranes were rinsed with Tris-Buffered Saline Tween (TBST) three times, and then the secondary antibody (ab6721, abcam) was added and maintained at 25°C for 1 hour on a shaker. Finally, the membranes were
EFFECTS OF KAEMPFEROL ON H9c2 CELLS

Results

OGD treatment suppressed cell viability and promoted cell apoptosis: IHD refers to a pathological state in which the blood perfusion and oxygen supply of the heart are reduced, resulting in abnormal myocardial energy metabolism and failure to support the normal work of the heart. In this study, we first conducted OGD treatment on H9c2 cells to construct a myocardial ischemia model. The results displayed in Figure 1A showed that the cell viability was significantly reduced by OGD treatment \( (P < 0.01) \), whereas the proportion of apoptotic cells was notably enhanced by OGD treatment \( (P < 0.001, \text{Figure 1B}) \). Meanwhile, the expression levels of apoptosis-associated proteins Cleaved-caspase-3, Cleaved-caspase-8, and Cleaved-caspase-9 were remarkably increased after OGD treatment, and the values of Cleaved/Pro-caspase-3, Cleaved/Pro-caspase-8, and Cleaved/Pro-caspase-9 were consequently increased compared with control \( (\text{all } P < 0.001, \text{Figure 1C, D}) \), which was coincident with the results of flow cytometry. These outcomes demonstrated that the myocardial ischemia model had been initially established.

Kae treatment attenuated OGD-induced cell damage: To illustrate the potential effects of Kae treatment on primary cardiomyocytes and H9c2 cells, we respectively pre-
Figure 2. Kae exposure attenuated OGD-induced cell damage. A and B: Kae exposure had no significant influence on H9c2 and primary cardiomyocytes cell viability, however, Kae pretreatment notably attenuated the inhibitory effects that OGD treatment induced on H9c2 and primary cardiomyocytes cell viability. C and D: Kae exposure have no significant influences on H9c2 and primary cardiomyocytes apoptosis, however, Kae pretreatment remarkably weakened the promoting effects that OGD treatment triggered on H9c2 and primary cardiomyocytes apoptosis. E-H: Kae exposure have no significant influences on the expression of apoptosis-related proteins including Cleaved-caspase-3, Cleaved-caspase-8 and Cleaved-caspase-9 in both H9c2 cells and primary cardiomyocytes, however, Kae pretreatment markedly diminished the promoting effects on the expression of apoptosis-related proteins induced by OGD treatment in these two cell lines. CTRL indicates control; ns, no significance; OGD, oxygen-glucose deprivation; and Kae, Kaempferol. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).
the role of miR-15b in the process of Kae attenuating the regulating miR-15b expression: Kae reduced OGD-induced cell damage by down-miR-15b expression. In other words, Kae pretreatment significantly reduced of apoptotic cells (both  

Figure 2A, B). In addition, data displayed in Figure 2C, D showed that Kae pretreatment markedly alleviated the promoting effects on cell apoptosis triggered by OGD treatment, which was exhibited as a decrease of the proportion of apoptotic cells (both P < 0.05). Meanwhile, the enhanced effects on the expression levels of apoptosis-associated proteins triggered by OGD treatment were remarkably weakened by Kae pretreatment, exhibiting as the ratios of Cleaved/Pro-caspase-3, Cleaved/Pro-caspase-8, and Cleaved/Pro-caspase-9 were dramatically reduced (all P < 0.01, Figure 2E-H). These outcomes indicated that Kae pretreatment itself had no significant influence on primary cardiomyocytes and H9c2 cells. However, it had notably alleviated the cell damages induced by OGD treatment.

Kae treatment reduced miR-15b expression: To probe into the underlying mechanisms of the aforementioned influences triggered by OGD and Kae, we detected the expression levels of miR-15b in non-treated, OGD-treated, Kae-treated, OGD + Kae-treated primary cardiomyocytes and H9c2 cells. The outcomes suggested that OGD treatment dramatically elevated the expression level of miR-15b (both P < 0.01, Figure 3A, B) in both primary cardiomyocytes and H9c2 cells, whereas Kae pretreatment remarkably weakened this elevating effect (both P < 0.05, Figure 3A, B). Besides, Kae treatment alone notably decreased the expression of miR-15b in both primary cardiomyocytes and H9c2 cells (both P < 0.05, Figure 3A, B). In other words, Kae pretreatment significantly reduced miR-15b expression.

Kae reduced OGD-induced cell damage by down-regulating miR-15b expression: For further investigating the role of miR-15b in the process of Kae attenuating the cell damages induced by OGD treatment, we first overexpressed miR-15b in OGD + Kae-treated primary cardiomyocytes and H9c2 cells by using cell transfection. Thereafter, cell viability and cell apoptosis were further examined. Data exhibited in Figure 4A, B revealed that miR-15b was exactly overexpressed in primary cardiomyocytes and H9c2 cells (both P < 0.01) and was notably silenced in primary cardiomyocytes (P < 0.01, Figure 4B). Besides, outcomes of CCK-8 assay specified that the promoting effects on cell viability evoked by Kae pretreatment in OGD-treated primary cardiomyocytes and H9c2 cells were notably counteracted by miR-15b mimic (both P < 0.05, Figure 4C, D). Moreover, flow cytometry outcomes revealed that miR-15b mimic notably reversed the inhibitory effects on cell apoptosis triggered by Kae pretreatment in OGD-treated these two cell lines (both P < 0.05, Figure 4E, F). Furthermore, the suppressive effects on the expression levels of cell apoptosis-associated factors including Cleaved/Pro-caspase-3, Cleaved/Pro-caspase-8, and Cleaved/Pro-caspase-9 triggered by Kae pretreatment in OGD-induced these two cell lines were attenuated by miR-15b mimic as well (P < 0.01 or P < 0.05, Figure 4G-J). All these data illustrated that Kae pretreatment attenuated OGD-induced cell damage through decreasing the expression of miR-15b and overexpression of miR-15b aggravated cell damage.

Bcl-2 and TLR4 were target genes of miR-15b: A previous investigation discovered that TLR4 was one of target genes of miR-15b-5p.29 Therefore, to uncover how miR-15b functioned in the process of Kae pretreatment attenuating the cell damages induced by OGD treatment, we explored the potential target genes. The results turned out to be that co-transfection of Bcl-2-MUT and miR-15b mimic had no significant influence on the luciferase activity, whereas it had notably reduced the luciferase activity of Bcl-2-WT and miR-15b mimic co-transfected group (P < 0.05, Figure 5A). Moreover, miR-15b mimic partially reversed the elevating effects on Bcl-2 expression evoked by Kae pretreatment in OGD-induced cells (P < 0.01, Figure 5B, C). Besides, co-transfection of TLR-4-WT and
Figure 4. Kae reduced OGD-triggered cell damage by down-regulating miR-15b expression. A: miR-15b was successfully overexpressed in H9c2 cells. B: miR-15b was successfully overexpressed and inhibited in primary cardiomyocytes. C and D: miR-15b mimic reversed the promoting effects on cell viability that Kae pretreatment had induced in OGD-treated H9c2 cells and primary cardiomyocytes. E and F: miR-15b mimic reversed the suppressive effects on cell apoptosis that Kae pretreatment induced in OGD-treated H9c2 cells and primary cardiomyocytes. G-J: miR-15b mimic partially reversed the inhibitory effects that Kae pretreatment evoked on the expression of apoptosis-related proteins including Cleaved-caspase-3, Cleaved-caspase-8 and Cleaved-caspase-9 in OGD-treated H9c2 cells and primary cardiomyocytes. CTRL indicates control; NC, negative control; OGD, oxygen-glucose deprivation; Kae, Kaempferol; and miR-15b, microRNA-15b. in, inhibitor. * P < 0.05, ** P < 0.01, *** P < 0.001.
miR-15b mimic also had remarkably declined the luciferase activity of TLR-4-WT group relative to NC mimic and TLR-4-WT co-transfected group (P < 0.05, Figure 5D). In addition, detection of the expression level of TLR4 revealed that miR-15b mimic markedly inhibited TLR4 expression; however, miR-15b in hibition dramatically promoted the TLR4 expression (both P < 0.01, Figure 5E, F). These results hinted that Bcl-2 and TLR4 were target genes of miR-15b.

**Kae regulated PI3K/AKT and Wnt/β-catenin pathways through down-regulating miR-15b expression:** To reveal the underlying mechanism, we examined the expression levels of key factors involved in the PI3K/AKT and Wnt/β-catenin pathways including p/t-PI3K, p/t-AKT, Wnt3a, and β-catenin. Outcomes displayed in Figure 6A-D illustrated that OGD treatment notably decreased the ratios of p/t-PI3K (P < 0.01, Figure 6A, B), p/t-AKT (P < 0.05, Figure 6A, B), Wnt3a (P < 0.01, Figure 6C, D) and β-catenin (P < 0.05, Figure 6C, D). However, Kae pretreatment significantly weakened these OGD-induced inhibitory effects (P < 0.05 or P < 0.01, Figure 6A-D). Moreover, miR-15b mimic reversed the elevating effects on the ratios of p/t-PI3K (P < 0.05, Figure 6A, B), p/t-AKT (P < 0.01, Figure 6A, B), Wnt3a (P < 0.05, Figure 6C, D) and β-catenin (P < 0.05, Figure 6C, D) induced by Kae pretreatment in OGD-treated cells.

**Discussion**

In this research, we first constructed a myocardial cell injury model by OGD treatment in primary cardiomyocytes and H9c2 cells. Besides, CCK-8 assay, flow cytometry, and western blot results demonstrated that Kae pretreatment diminished OGD-evoked cell injury and reduced the expression of miR-15b. Moreover, miR-15b mimic reversed Kae-induced effects. Meanwhile, dual-luciferase activity assay results indicated that Bcl-2 and TLR4 were downstream target genes of miR-15b.

IHD is triggered by decreased blood supply to the heart, leading to insufficient oxygen and energy delivered to the myocardium. To simulate the state of cells under IHD, we performed OGD treatment on H9c2 cells. The result, which were consistent with previous studies displayed that OGD treatment remarkably decreased cell viability, whereas it accelerated cell apoptosis.30-32) Quantities of earlier studies have clarified the protective effects of Kae against cardiac injuries evoked by I/R, anoxia/reoxygenation, or hyperglycemia.16,17,53) Previous studies performed by Kim, et al. and Zhou, et al. revealed that Kae exposure attenuated I/R treatment-triggered sup-
pressive effects on cell viability and repressed the promoting effects on cell apoptosis; also, the elevated expression level of Caspase-3 was decreased by Kae exposure. Besides, the same changes evoked by Kae occurred in anoxia/reoxygenation-triggered myocardial cells. To illuminate the influences of Kae on OGD-evoked H9c2 cell injuries, we pretreated the OGD-induced H9c2 cells with Kae. The results suggested that OGD-induced inhibitory effects on cell viability was markedly diminished by Kae pretreatment. Besides, the promoting effects on cell apoptosis were alleviated by Kae pretreatment, which was verified by the outcomes of western blot, exhibiting that the elevated expression of apoptosis-associated proteins including Cleaved-caspase-3, Cleaved-caspase-8 and Cleaved-caspase-9 were all dramatically mitigated by Kae pretreatment. These outcomes were in line with the findings of aforementioned researches.

It has been well acknowledged that miRNAs play an important role in the protection of myocardial cells against cell damages evoked by I/R or hypoxia/reoxygenation. The expression of miR-15b was reported to be closely related to I/R treatment and cell apoptosis. To determine whether a correlation existed between Kae pretreatment and miR-15b expression, we detected the expression of miR-15b in Kae-exposed H9c2 cells. The results turned out to be that the expression of miR-15b was remarkably decreased by Kae pretreatment in OGD-induced H9c2 cells. To uncover how miR-15b participated in the process of Kae regulating OGD-induced cell injuries, we overexpressed miR-15b in Kae+ OGD-treated H9c2 cells. The results suggested that miR-15b mimic inhibited the promoting effects triggered by Kae pretreatment, whereas it relieved the suppressive effects induced by Kae pretreatment. The expression variation of apoptosis-associated proteins including Cleaved-caspase-3, Cleaved-caspase-8 and Cleaved-caspase-9 examined by western blot further verified the above-mentioned results. These outcomes indicated that Kae pretreatment weakened OGD-induced cell injuries by reducing the expression of miR-15b.

Because OGD-triggered cell injuries were mainly reflected in the promotion of cell apoptosis, we speculated...
that miR-15b may work via targeting apoptosis-related factors. While earlier studies conducted in cardiomyocyte and Sprague-Dawley (SD) rats suffering from focal cerebral ischemia pointed out that miR-15b exerted its regulatory effects on cell apoptosis via targeting Bcl-2. \(^{24,27}\) The results of dual-luciferase activity assay and western blot conducted in this study exactly affirmed the aforementioned findings.

Plenty of previous researches have clarified the participation of PI3K/AKT and Wnt/\(\beta\)-catenin pathways in the protection of myocardial cells. For instance, Matsui, et al. declared that PI3K/AKT pathway played a role in assisting ischemia-injured cardiomyocyte survival. \(^{30}\) Besides Zhang, et al. demonstrated that sulfiredoxin-1 exerted its protective effects on cardiomyocyte via suppressing mitochondrial apoptosis through activating PI3K/AKT pathway. \(^{31}\) Moreover, Lin, et al. demonstrated that trimethylpyrazine improved hypoxia-evoked injury in myocardial cells via up-regulating PI3K/AKT pathway. \(^{36}\) In addition to PI3K/AKT pathway, Ozhan, et al. reported that Wnt/\(\beta\)-catenin pathway play key roles in the progress of cell apoptosis and survival of myocardial cell. \(^{37}\) Besides, Cui, et al. revealed that the exosomes derived from adipose-derived mesenchymal stem cells protected myocardium from I/R injury via activating Wnt/\(\beta\)-catenin pathway. \(^{38}\) In this study, we observed that Kae pretreatment prominently elevated the expression level of the key pathways including p/t-PI3K, p/t-AKT, Wnt3a and \(\beta\)-catenin pathways by decreasing the expression of miR-15b and thereby activates PI3K/AKT and Wnt/\(\beta\)-catenin pathways by decreasing the expression of miR-15b.

Conclusion

In conclusion, this investigation indicated that Kae alleviated OGD-induced cell injury through down-regulating the expression of miR-15b and thereby activating PI3K/AKT and Wnt/\(\beta\)-catenin pathways.

Disclosure

Conflicts of interest: None.

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