Kaempferol reversals retinal ischemia/reperfusion (IR) injury through activating of PI3K/Akt/mTOR signaling pathway

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Research

Keywords: Retinal ischemia/reperfusion, Kaempferol, Apoptosis, Inflammation

DOI: https://doi.org/10.21203/rs.3.rs-258600/v1

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Abstract

Purpose Retinal ischemia/reperfusion (IR) injury is associated with many ocular diseases, including acute glaucoma, diabetic retinopathy, and retinal vascular occlusion. However, currently there are no effective medications to prevent the development of retinal IR injury. Kaempferol is a kind of plant extract which has showed an excellent ability to inhibit the inflammation. Materials and Methods In this study, both in vitro and in vivo retinal oxidative damage models were established. Cell viability was assessed by Cell Counting Kit-8 assay. Apoptosis was examined using flow cytometry analysis. Atherosclerotic lesion analysis was performed using hematoxylin-eosin staining. The expressions of inflammatory cytokines were detected by quantitative real-time PCR and ELISA respectively. The effect of expression of autophagy and the PI3K/Akt/mTOR signaling pathway related pathway was evaluated by Western blot. Results We found kaempferol was able to protect the viability of ARPE-19 cells against oxidative damage by reducing its apoptosis. In addition, it also kept structurally complete epithelium, stroma and endothelium of cornea after oxidative damage. Moreover, it also able to reduce the expression of inflammatory cytokines and increased the expression of anti-inflammatory cytokines. Kaempferol was able to enhanced the expression of anti-apoptotic genes BCL-2, and reduced the expression of autophagy gene Beclin 1 and increased the expression of anti-autophagy gene LC-3, was also able to enhance the expression PI3K and the phosphorylation of Akt and mTOR. Conclusion Kaempferol reversals retinal ischemia/reperfusion (IR) injury through activating of PI3K/Akt/mTOR signaling pathway.

Introduction

Retinal ischemia / reperfusion (IR) injury involves many ophthalmic diseases, such as diabetic retinopathy, ocular ischemic syndrome, glaucoma and ischemic optic neuropathy, which can lead to irreversible neuronal cell damage. Studies speculate that oxidative stress may be related to IR-induced retinal damage. Oxidative stress is defined as an increase in the physiological value of intracellular reactive oxygen species (ROS) concentration. ROS includes molecules such as singlet oxygen, hydroxyl radical, hydrogen peroxide, peroxy radical, nitric oxide and superoxide anion. When the endogenous activity of antioxidant enzymes (such as glutathione, catalase, superoxide dismutase, glutathione, metallothionein and SOD) and/or the concentration of certain vitamins changes, this will happen. A large amount of evidence indicates that ROS is the initial cause of IR damage. Thus, lots of studies focused on reducing the ROS. It is well known that the ROS was able to induce inflammatory responses. So, our study is trying to targeting the inflammation.

Lots of plant extracts have showed great potential on anti-inflammation. Kaempferol, 3,4′,5,7-tetrahydroxyflavone, is a natural flavonol, a type of flavonoid, found in a variety of plants and plant-derived foods including kale, beans, tea, spinach and broccoli. Kong et al. reported kaempferol showed an anti-inflammatory effect on early atherosclerosis in high cholesterol fed rabbits. Similarly, Park et al. revealed the kaempferol also had anti-inflammatory effect inaged kidney tissues by inhibiting the nuclear factor-κB via nuclear factor-inducing kinase/IKBkinase and mitogen-activated protein kinase pathways. However, the kaempferol doesn’t use for the treatment of oxidative stress injury of the retina. Our
hypothesis that the kaempferol could be used for the treatment of oxidative stress injury of the retina by reducing inflammatory responses. In this study, both in vitro and in vivo oxidative stress injury model was established. The protective effects of kaempferol were explored in this study, and we also explored the potential mechanism.

Materials And Methods

**ARPE-19 cell culture and oxidative injury model**

The ARPE-19 cell line (CRL-2302, ATCC) was maintained in DMEM/12 medium supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin. The cells incubated at 37 °C and 5% CO₂. The in vitro oxidative injury model of ARPE-19 cells was established by treated with 1 mM H₂O₂ for 24 h. ARPE-19 cells proliferation was detected using Cell Counting Kit-8 (Sigma-Aldrich, St. Louis, USA) under the instructions of the manufacturer. Then the absorbance values were detected at different time points (450 nm). All experiments were performed three times.

**Cell apoptosis assay**

ARPE-19 cells were detached from the culture plate by trypsin and washed with PBS. Then, cells were centrifuged and stained with annexin V-FITC and propidium iodide (PI) (Sigma, St. Louis, USA) and for 20 minutes at room temperature. Finally, the apoptosis of cells was evaluated by flow cytometry. The apoptosis test was repeated three times.

**H&E staining**

After the hearts were harvested, samples were washed with PBS and fixed in 4% PFA. Tissues were dehydrated in a series of graded ethanol solutions (70–100%) and embedded in paraffin. Five-micrometer sections were prepared and stained with either Hematoxylin and Eosin (H&E) staining according to the standard procedures.

**ELISA assay**

The tissues were washed with PBS twice and tissue homogenate was made according to the instructions of manufacturer at 4 °C, then centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was used for ELISA detection. The secretion level of VEGF, IL-10 and IFN-γ were detected using a commercially available enzyme-linked immunosorbent assay (ELISA) system (Lichen, Shanghai, China) following kit instructions.

**RT-PCR**

Total RNAs were isolated from cell lysis solution by using a commercial RNA extraction kit, and the RNA concentration was measured by nanodrop. Fifteen microliters of RNA (50ng/ul) was used as a template to prepare for the cDNA. The relative expression of IL-1, TNF-α, TLR-4, NK-kB was quantified using SYBR
green reagents on the ABI 7500 sequence detection system. PCR was performed with the following thermocycling conditions: An initial 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The primers were purchased from Funengbio Co. (Shanghai, China). Housekeeping gene β-actin was used as an internal reference to normalize the results. For the analysis of the results, the $2^{-\Delta\Delta Ct}$ method was performed to calculate the relative expression.

Western blotting

The ARPE-19 cells were lysed directly for 5 minutes in the Lysis buffer. Lysates were separated by centrifugation (13,000×g, 30 min, 4 °C) and 50 µg of total proteins was electrophoresed on a 10% SDS-PAGE, which was transferred onto polyvinylidene difluoride (PVDF) membranes in a transfer tank using transfer buffer (195 mM glycine, 25 mM Tris-HCl and 20% (v/v) methanol). The first stained membrane was confirmed the transfer efficiency with Ponceau S. Then the PVDF membranes were blocked for 1 h at RT with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (50 mM Tris, pH 8.0, 150 mM NaCl) with 0.05% Tween 20 (TBS-T). Membranes were incubated by primary antibody diluted 1:1000, then followed by secondary antibody conjugated with horseradish peroxidase at 1:2000 dilutions. Positive band intensities were shown by utilizing a gel documentation system (LAS-3000 Fujifilm).

Statistical analysis

In this study, all results were exhibited as the mean ± S.D. And the statistical analysis was performed using GraphPad Prism 8.0 software. Differences between two groups were evaluated using student T-test, and differences among the groups were assessed using one-way ANOVA. A p-value <0.05 was considered statistically significant.

Results

Kaempferol protects the viability of ARPE-19 cells against oxidative injury

The chemical formula of kaempferol was shown in Figure 1A. The viability of ARPE-19 cells was significantly reduced after treated with H$_2$O$_2$. However, the viability of H$_2$O$_2$ pretreated ARPE-19 cells gradually increased with the treatment of increased kaempferol from 1mM to 10mM (Figure 1B). Suggesting the kaempferol has protective role on ARPE-19 cells against oxidative injury.

Kaempferol reduces the apoptosis of ARPE-19 cells against oxidative injury

Following, the apoptosis behaviors of ARPE-19 cells with different treatment were explored. As shown in Figure 2, the apoptotic rate of ARPE-19 cells treated with H2O2 was significantly increased to 13.73% compared to the control 2.65%. The apoptotic rate of ARPE-19 cells was reduced to 8.17% with the treatment of 1mM kaempferol. And it further decreased to 6.22% and 4.59% with the treatment of 5 mM and 10 mM kaempferol respectively. This result revealed that kaempferol could reduces the apoptosis of ARPE-19 cells after oxidative injury.
Kaempferol keeps structural and organizational integrity of cornea after oxidative injury.

As shown in Figure 3, structurally complete epithelium, stroma and endothelium of cornea was exhibited in the control group. However, the epithelium was disappeared and the structure of stroma became loose after the cornea treated with H$_2$O$_2$. Fortunately, the epithelium came back after treated with 50 mg/kg kaempferol. The treatment of 12.5 mg/kg and 25 mg/kg kaempferol failed to regenerated the damaged cornea.

The anti-inflammatory effects of kaempferol

Then we explored the inflammatory responses after kaempferol treatment. As shown in Figure 4A-4C, the secretion of VEGF and IFN-$\gamma$ of cornea treated with H$_2$O$_2$ was significantly higher than the control group. And it gradually decreased with the treatment of kaempferol, and the higher concentration of kaempferol showed lower secretion of VEGF and IFN-$\gamma$. On the contrary, the secretion of IL-10 of cornea treated with H$_2$O$_2$ was significantly reduced compared to the control group. And it gradually increased with the treatment of kaempferol, and the higher concentration of kaempferol showed higher secretion of IL-10. Moreover, the RT-PCR results showed that the relative expression of inflammatory cytokines, including IL-1$\alpha$, IL-6 and TNF-$\alpha$ were significantly reduced in kaempferol treated groups. The pro-inflammatory effects of kaempferol was in a dose depend manner. While the relative expression of IL-10 was gradually increased with the increased concentration of kaempferol (Figure 4D).

The potential mechanism of the protective effects of kaempferol

Finally, we also explored the potential mechanism of the protective effects of kaempferol on cornea against oxidative injury. As shown in Figure 5, firstly, we examined the expression of apoptosis and autophagy related gene expression. We found kaempferol was able to enhanced the expression of anti-apoptotic genes BCL-2, and reduced the expression of autophagy gene Beclin 1 and increased the expression of anti-autophagy gene LC-3. In addition, we also found kaempferol was also able to enhance the expression PI3K and the phosphorylation of Akt and mTOR. Suggesting the protective effects of kaempferol against oxidative injury via activating the PI3K/Akt/mTOR signaling pathway.

Discussion

The PI3K/AKT/mTOR pathway is an intracellular signaling pathway important in regulating the cell cycle. Therefore, it is directly related to cellular quiescence, proliferation, cancer, and longevity. PI3K activation phosphorylates and activates AKT, localizing it in the plasma membrane. AKT can have a number of downstream effects such as activating CREB, inhibiting p27, localizing FOXO in the cytoplasm, activating PtdIns-3ps, and activating Mtor which can affect transcription of p70 or 4EBP1. In many cancers, this pathway is overactive, thus reducing apoptosis and allowing proliferation. This pathway is necessary, however, to promote growth and proliferation over differentiation of adult stem cells, neural stem cells specifically. It is the difficulty in finding an appropriate amount of proliferation versus differentiation that researchers are trying to determine in order to utilize this balance.
in the development of various therapies. Additionally, this pathway has been found to be a necessary component in neural long-term potentiation. In this study, we demonstrated that kaempferol protected cornea against oxidative injury via activating the PI3K/Akt/mTOR signaling pathway.

In our study, we first demonstrated that the kaempferol was able to reverse the viability of ARPE-19 cells after oxidative damage by reducing cell apoptosis. Kimura et al. reported glucagon-like peptide-1 (GLP-1) protects against methylglyoxal-induced PC12 cell apoptosis through enhancing the PI3K/Akt/mTOR signaling pathway, which was consistent with our study, the PI3K/Akt/mTOR signaling pathway could prevent cells from apoptosis. Moreover, we also found the kaempferol was capable of reducing the expression of inflammatory cytokines and increasing the expression of anti-inflammatory cytokines. Wu et al. revealed that gambogic acid suppresses inflammation in rheumatoid arthritis rats via up-regulating the PI3K/Akt/mTOR signaling pathway. Our results were consistent with this study, the up-regulating the PI3K/Akt/mTOR signaling pathway could decreased the inflammation. According to the abovementioned information, our study provides a promising strategy to protect the eyes against from oxidative injury.

Conclusion

Kaempferol reversals retinal ischemia/reperfusion (IR) injury through activating of PI3K/Akt/mTOR signaling pathway.

Abbreviations

IR : Retinal ischemia / reperfusion
ROS : reactive oxygen species
FBS : fetal bovine serum
PBS : phosphate buffer saline
PFA : Paraformaldehyde Fix Solution
H&E staining: hematoxylin-eosin staining
ELISA: enzyme linked immunosorbent assay
RT-PCR : Reverse Transcription-Polymerase Chain Reaction

Declarations

Acknowledgments

The authors thank all students and technicians in the laboratory for their cooperation.
Funding
Not applicable.

Authors' contributions
LLL conceived and designed the experiments; DS, DGC, SFS and WWW performed the experiments; DS and SFS analyzed the data; SD, SFS and LLL wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
The data and materials used in the current study are all available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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