Kaempferol-induced GPER upregulation attenuates atherosclerosis via the PI3K/AKT/Nrf2 pathway

Zhuo Feng, Changyuan Wang, Yue, Jin, Qiang Meng, Jingjing Wu and Huijun Sun

The First Affiliated Hospital of Dalian Medical University, Dalian, China; Academy of Integrative Medicine, Dalian Medical University, Dalian, China; Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, Dalian, China

ABSTRACT

Context: The effect of kaempferol, a regulator of oestrogen receptors, on atherosclerosis (AS) and the underlying mechanism is elusive.

Objective: To explore the effect and mechanism of kaempferol on AS.

Methods and materials: In vivo, C57BL/6 and apolipoprotein E (APOE)−/− mice were randomly categorized into six groups (C57BL/6: control, ovariectomy (OVX), high-fat diet (HFD); APOE−/−: OVX-HFD, APOE-HFD + kaempferol (50 mg/kg) and OVX-HFD + kaempferol (100 mg/kg) and administered with kaempferol for 16 weeks, intragastrically. Oil-Red and haematoxylin–eosin (HE) staining were employed to examine the effect of kaempferol. In vitro, human aortic endothelial cells (HAECs) were pre-treated with or without kaempferol (5, 10 or 20 μM), followed by administration with kaempferol and oxidized low-density lipoprotein (ox-LDL) (200 μg/mL). The effect of kaempferol was evaluated using flow cytometry, and TdT-mediated dUTP Nick-End Labelling (TUNEL).

Results: In vivo, kaempferol (50 and 100 mg/kg) normalized the morphology of blood vessels and lipid levels and suppressed inflammation and apoptosis. It also activated the G protein-coupled oestrogen receptor (GPER) and PI3K/AKT/nuclear factor-erythroid 2-related factor 2 (Nrf2) pathways. In vitro, ox-LDL (200 μg/mL) reduced the cell viability to 50% (IC50). Kaempferol (5, 10 or 20 μM) induced-GPER activation increased cell viability to nearly 10%, 19.8%, and decreased cellular reactive oxygen species (ROS) generation (16.7%, 25.6%, 31.1%), respectively, consequently attenuating postmenopausal AS. However, the protective effects of kaempferol were blocked through co-treatment with si-GPER.

Conclusions: The beneficial effects of kaempferol against postmenopausal AS are associated with the PI3K/AKT/Nrf2 pathways, mediated by the activation of GPER.

Introduction

Globally, most deaths from coronary heart disease are associated with atherosclerosis (AS) (Wang C et al. 2018). Although AS occurs in both men and women, reports from epidemiological studies show that postmenopausal women exhibit a higher prevalence of atherosclerotic disease than premenopausal women, suggesting the potential role of oestrogen in the progression of atherosclerotic disease (Aryan et al. 2020). Oestrogen is essential in the regulation of lipid metabolism (Morselli et al. 2018), inflammation (Mu et al. 2016) and vascular homeostasis (Filardo 2018). Natural oestrogen or selective oestrogen receptor (ER) modulators are crucial in cardiovascular diseases as they regulate the lipid level in blood vessels.

In clinics, AS patients are managed with statins (Okuyama et al. 2015), aspirin (Petri et al. 2017), nitroglycerin (Cho et al. 2011) and other drugs (Li et al. 2019). The lack of oestrogen is attributed to the loss of its protective effects in postmenopausal women, for example, anti-inflammation, antioxidant, low blood lipids, cholesterol, etc., subsequently contributing to the high incidence of AS. Therefore, oestrogen replacement therapy is administered as the basic clinical treatment of postmenopausal AS. Although this management option achieves a particular short-term effect, some long-term side effects occur, including fatigue, uterine leiomyoma and canceration (Malekmohammad et al. 2019). Accordingly, researchers have increasingly shifted their focus to explore safer and alternative strategies for managing AS. In China, traditional Chinese medicines (TCM) have been extensively adopted to manage various diseases for thousands of years (Hu et al. 2019; Jian et al. 2019). TCM exerts excellent effects in clinical AS therapy (McClelland et al. 2015; Zhu et al. 2020) and has attracted considerable attention from scholars. TCM contains phytoestrogen, with numerous pharmacological effects, and has been applied for the therapeutic management of human diseases (Kiyama 2017). Recent exploration of anti-AS mechanisms of phytoestrogen has uncovered more active ingredients which can be used to synthesize better therapeutic drugs (Gencel et al. 2012).

Oestrogens, produced in endocrine organs and reproductive systems, play a vital role in humans and animals. Phytoestrogens, as a family of exogenous oestrogens, widely exist in plants and food and are considered a promising therapeutic target for postmenopausal atherosclerotic (Morselli et al. 2017). Kaempferol, another natural flavonoid, is widely distributed in...
plants and possesses various pharmacological activities, including anti-inflammatory, anti-apoptotic and anticancer effects (Zhong et al. 2018; Sharma et al. 2019). Notably, it plays a vital role in cancer therapy (Kim et al. 2018; Wu et al. 2018) and is a therapeutic agent for various diseases, including diabetes and obesity (Alkhaldi et al. 2018; Torres-Villarreal et al. 2019). Recent evidence indicates that kaempferol alleviates oxidized low-density lipoprotein (ox-LDL)-induced endothelial cell injury, associated with the inflammation-dependent pathway (Che et al. 2017). Another study in rats found that kaempferol exerted therapeutic effects on AS (Zhang et al. 2016). However, additional studies are warranted to certify the anti-postmenopausal atherosclerotic effect of kaempferol.

Compelling evidence confirmed that membrane G protein-coupled oestrogen receptor (GPER), a newly discovered ER activated by classic oestrogen and phytoestrogens, is associated with numerous diseases (Barton and Prossnitz 2015; Grande et al. 2020). In particular, the expression of GPER modulated by the regulator changed the concentration of oestrogen and further affected the deterioration of AS in postmenopausal women. In addition, GPER regulators stimulate the PI3K signal pathway to fight various diseases (Fan et al. 2018); this pathway is associated with the expression level of oestrogen (Pollard and Daniel 2019). Notably, PI3K/AKT/Nrf2 pathway is the most widely studied. However, whether kaempferol, as an activator of GPER, attenuates AS via modulation of the PI3K/AKT/Nrf2 signalling pathway is elusive.

In this article, we revealed the effects of kaempferol on postmenopausal AS in ovariectomy (OVX)+high-fat diet-induced apolipoprotein E (APOE)−/− mice and in ox-LDL induced human aortic endothelial cells (HAEcs) and explored the underlying molecular mechanisms. Also, we have elucidated the role of GPER in postmenopausal AS.

**Materials and methods**

**Reagents**

HAEcs were purchased from Shanghai Bioleaf Biotech Co., Ltd. (Shanghai, China). The HAEcs medium, Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were acquired from the Gibco-BRP Company (Gaithersburg, MD). Kaempferol (purity >98%) was purchased from Meilun Biotechnology Co. Ltd. (Dalian, China). Fresh plasma was acquired from the blood bank. For the cell viability test, we employed the Cell Count Kit-8 (CCK-8) assay, provided by Proteintech Group (Wuhan, China). TUNEL Staining Kit, purchased from Beyotime Biotechnology (Haimen, China), was employed to assess the level of cell apoptosis. Intracellular ROS was estimated using the ROS Assay Kit (Beyotime Biotechnology, Haimen, China). Antibodies specific for Bax, B-cell lymphoma-2 (Bcl-2), caspase 3, AKT, P-AKT, heme oxygenase-1 (HO-1), Nrf2, PI3K, intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were obtained from the Proteintech Group (Wuhan, China), whereas antibody against GPER was purchased from BOSTER Biological Technology (Pleasanton, CA). The BCA Protein Assay Kit was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Histopathology test was conducted using the Oil Red staining kit, Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

**Animal experiments**

Eight-week-old female C57BL/6 mice were obtained from the Laboratory Animal Center of Dalian Medical University (Dalian, China). APOE−/− mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal studies adhered to the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. Approval was issued by the ethics committee of Dalian Medical University. The living space had controlled temperature and a 12 h light/dark cycle. Mice accessed free water and were fed on 6 g normal food or high-fat diet (HFD, purchased from Jiangsu Medicine Ltd. (Lianyungang, China), containing 1.25% cholesterol and 0.5% sodium cholate. After one-week of adaptive feeding, C57BL/6 mice were randomly categorized into three groups, n = 8 for per group: A (control), B (OVX), C (HFD); APOE−/− mice were randomly divided into three groups, n = 8 for per group: D (OVX-HFD), E (OVX-HFD + kaempferol 50 mg/kg) and F (OVX-HFD + kaempferol 100 mg/kg), and then subjected to bilateral OVX or sham OVX (sham OVX). The mice in groups A, B, C and D were intragastrically administered with 0.5% CMC-Na per day, whereas mice in groups E and F were given kaempferol (dissolved with 0.5% CMC-Na) via the same route. After 16 weeks, all mice were anaesthetized using 4% chloral hydrate (Sigma, St. Louis, MO). Then, blood samples were drawn from the abdominal aorta. The aorta tissues were collected for further analysis.

Method of OVX: the mice were fixed with prone position, dorsal skin depilated and iodophor disinfected. The skin and muscle layer were cut and the fat tissue was clipped with tweezers and pull it out the wound. Further, the blood vessels were ligated at the connection between the fallopian tube and uterine horn was cut and the ovary moved out. Next, ovaries and surrounding fat tissue were removed, the incision was closed by suturing the muscles and stapling the skin. Finally, mice were given intramuscular injection of penicillin.

**Oil red O staining**

For tissue preparation, a longitudinal cut was made in the aortic arch, thoracic and abdominal aortae, and pinned flat. After rinsing in 60% isopropanol and PBS, tissues were stained with oil red O. We calculated the ratio of oil red O positive staining to the total surface area to estimate the face lesion size.

**Haematoxylin–eosin (HE) staining**

Arterial tissues for atherosclerotic lesions morphometric analysis were immersed in 10% formalin for 30 min, dehydrated in 75% ethanol overnight. The tissues were then embedded in paraffin and sliced into 4 μm sections. Haematoxylin and eosin-stained sections were subjected to histopathological examination.

**Biochemical analysis**

Plasma samples were centrifuged at 3000 rpm at 4 °C for 10 min and preserved at −80 °C, awaiting subsequent analysis. The levels of serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDL-C), superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) were assessed using commercial kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).
Enzyme-linked immunosorbent assay (ELISA)

HAECs were seeded and cultured in the six-well plates and then treated with indicated drugs (kaempferol and/or ox-LDL). Cells were collected in a lysate buffer. Plasma was centrifuged (2000 rpm, 4°C, 5 min). The levels of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured using TNF-α and IL-6 ELISA kits (Huamei, Wuhan, China) in serum and cells. The values of cell TNF-α and IL-6 were normalized based on cell protein concentrations assessed using the BCA Protein Assay Kit.

Western blot

Protein samples were extracted from aorta tissues or HAECs lysates according to the protein extraction kit (Nanjing Jiancheng, Nanjing, China). Extracts were subjected to sodium dodecyl sulphate-polyacrylamide mini gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked using 5% milk for 2 h and then incubated with specific primary antibodies at 4°C overnight. This was followed by three times wash and continuous incubation with corresponding secondary antibodies at room temperature for 2 h. Eventually, a Bio-Rad imaging system with a Chemi HR camera 410 was used to capture the emitted light from the membranes exposed to enhanced chemiluminescence plus reagents (Beyotime, Haimen, China). The density of bands was analysed using Gel-Pro Analyser Version 4.0 (Media Cybernetics, Rockville, MD).

Cell culture and experiment design

HAECs were cultured in DMEM containing 10% FBS (Gibco, Gaithersburg, MD) in the incubator under the following conditions: humidity, 95%; temperature, 37°C; CO2, 5%. All examinations of HAECs were conducted at 24 h after replenishing the common medium with the basal serum-free medium containing kaempferol (5, 10 or 20 μM) or ox-LDL (200 μg/mL, final).

Preparation of ox-LDL

To isolate the plasma, the blood sample was centrifuged for 5 min at 7000 rpm and 4°C. The plasma was further subjected to discontinuous density-gradient hypervelocity centrifugation with Beckman Coulter Optima l-100 XP super centrifuge. It was then dialysed in phosphate-buffered saline (PBS) at 4°C for 24 h to prepare the original low-density lipoprotein (LDL, density: 1.019–1.063 g/mL). Original LDL was oxidized in the presence of 50 μM CuSO4 at 37°C for 24 h and terminated with 0.01% EDTA in PBS and sterilized via filtration (0.22 μm). Ox-LDL was preserved in the dark and prepared every two weeks.

CCK-8 assay

HAECs seeded into the 96-well plate to a density of 1 × 10^5 cells/mL were then treated with specific drugs and grown to approximately 80% confluence. The complete medium was replenished with serum-free medium containing ox-LDL (0–300 μg/mL), kaempferol (0–200 μM) or combining ox-LDL (200 μg/mL) with kaempferol (0–20 μM). After 24 h incubation, 10 μL CCK-8 solution was added and further incubated for 1.5 h at 37°C. A microplate reader (Tecan, Grödig, Austria) was used to measure the absorbance at 450 nm. The Prism GraphPad was employed to calculate the IC_{50} values (La Jolla, CA).

Cell apoptosis assay

HAECs cells were seeded and cultured in six-well plates. After treatment with indicated drugs, cells were collected to evaluate the apoptotic level using TUNEL Staining Kit. Briefly, the apoptotic rate was calculated as apoptotic cells/total cells, which emitted green and blue fluorescence, respectively, under an inverted fluorescence microscope.

Intracellular ROS assay

We applied 2',7'-dichlorodihydrofluorescein diacetates (DCFH-DA) as the probe to estimate intracellular reactive oxygen species (ROS) of cells incubated with ox-LDL (200 μg/mL) or ox-LDL combined with kaempferol (5, 10 and 20 μM). Briefly, after incubation with indicated drugs, HAECs were further incubated with a serum-free medium containing DCFH-DA (10 μM) at 37°C for 30 min. The BD FACS Calibur system (Becton, Dickinson and Company, Franklin Lakes, NJ) was employed to evaluate the fluorescence intensity with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

siRNA transfection

The plasmid DNA and Lipofectamine 2000 were premixed in DMEM for 20 min. HAECs were left to grow to 50% confluence, and then GPER siRNA (20 nM) with Lipofectamine 2000 was added to complete the transfection. The medium was replenished with a serum-free medium at 6 h post transfection.si-RNA sequence: GCTGTACATTGAGCAGAAA.

Statistical analysis

All results were presented as the mean ± SD from at least three independent experiments. The data were analysed using the Prism program (Version 6.0, GraphPad, San Diego, CA). Statistical analysis was performed using one-way ANOVA followed by Tukey’s post hoc test or unpaired t-test. p Values <0.05 denoted statistically significant differences.

Results

Effects of kaempferol on the vasculature and lipid homeostasis in HFD-OVX induced APOE<sup>−/−</sup> mice

Oil red O staining revealed a larger lesion area of mice in the HFD-OVX group compared to mice in OVX, HFD and normal groups. Besides, kaempferol treatment significantly reduced the lesion area and thickness (Figure 1(A)). HE staining results demonstrated that the intimal lesion area contained larger necrotic cores in the aorta at the HFD-OVX group, whereas kaempferol administration potentially reduced the lesion areas (Figure 1(B)). These observations confirmed the inhibitory effects of kaempferol on the progression of atherosclerotic lesions.

Based on the assay of levels of serum TC, LDLc, HDLc and TG, we examined the effect of kaempferol on lipid levels. Higher serum levels of TC, LDLc and TG were found in the HFD-OVX group compared to OVX, HFD and normal groups (Figure 2). In the groups treated with...
kaempferol, these changes were remarkably reversed compared to the HFD-OVX group.

**Effects of kaempferol on the biochemical indices and inflammatory factors in HFD-OVX induced APOE<sup>−/−</sup> mice**

Oxidative stress is recognized as one of the pivotal factors of the AS pathological process. With kaempferol treatment, higher levels of serum SOD and GSH and lower serum level of MDA were reported compared to the HFD-OVX group (Figure 3(A–C)); also, it substantially lowered serum TNF-α and IL-6 levels compared to the HFD-OVX group. Western blot results demonstrated that the protein expression of ICAM and VCAM was significantly down-regulated by kaempferol (Figure 3(F–H)). Overall, these findings provide insights that kaempferol significantly lowered inflammation and inhibit oxidative stress.
Effects of kaempferol treatment on apoptosis in HFD-OVX induced APOE\(^{-/-}\) mice

It has been widely confirmed that apoptosis of HAECs promoted the initiation and progression of AS by forming necrotic cores (Yang et al. 2019). Thus, the expression of apoptosis-related protein Bax, Bcl-2 and caspase 3 was assessed. In the HFD-OVX group, the ratio of Bax/Bcl-2 and expression level of caspase 3 were higher compared to OVX, HFD and normal groups (Figure 4), whereas kaempferol treatment markedly reversed this tendency. Kaempferol (100 mg/kg) induced a 2.65-fold and 2.1-fold reduction in the expression of Bax/Bcl-2 and caspase 3, respectively, compared to the OVX-HFD group. Thus, we concluded that kaempferol-mediated reduction in apoptosis potentially improves the condition of AS.

Effects of kaempferol treatment on GPER expression and PI3K/AKT/Nrf2 pathway in HFD-OVX induced APOE\(^{-/-}\) mice

Recent findings suggested that GPER was involved in the progression of AS (Aryan et al. 2020). To explore the relationship between the beneficial effect of kaempferol on AS and GPER expression as well as PI3K/AKT/Nrf2 pathway. The protein expressions of GPER, PI3K, Nrf-2 and HO-1 were measured by western blotting \textit{in vivo}. The level of GPER expression was markedly decreased in OVX and OXV-HFD groups compared to the control group; however, no significant change was reported in the HFD group. Compared to the OXV-HFD group, kaempferol administration was associated with a significantly higher expression of GPER (Figure 5(A)). Similarly, down-regulatory effects of expression of PI3K, Nrf-2, HO-1 and P-AKT/AKT
were observed in OVX, HFD and OVX-HFD groups. Notably, kaempferol (50 and 100 mg/kg) induced remarkably high expression of the above proteins (Figure 5(B–F)). Collectively, these findings provide evidence on the potential benefits of kaempferol on AS through upregulation of GPER expression and activation of the PI3K/AKT/Nrf2 pathway.

Effects of kaempferol on cell viability and apoptosis in HAECs treated with ox-LDL

We performed a CCK-8 kit assay to detect the effect of kaempferol on cell viability in HAECs. Notably, the cell viability decreased with elevated concentrations of ox-LDL (Figure 6(A)); the IC50 of ox-LDL to the cell was approximated at 200 μg/mL. Moreover, kaempferol (concentration range, 0–20 μM) did not exert any toxicity effect on HAECs cells but exhibited a beneficial effect on cell viability in HAECs (Figure 6(B,C)). Thus, we applied 200 μg/mL ox-LDL and 5, 10 and 20 μM kaempferol for the follow-up experiments. Evaluation of the effect of kaempferol on cell apoptosis via TUNEL staining revealed few TUNEL-positive cells in the control group and kaempferol alone group. However, ox-LDL induced the generation of numerous apoptosis cells (Figure 7(A)). Notably, compared to the ox-LDL group, co-administration with kaempferol (5, 10 and 20 μM) significantly lowered apoptosis. We further evaluated the expression of apoptotic-related proteins via Western blotting to assess the antiapoptotic effects of kaempferol on HAECs. Of note, ox-LDL administration induced higher expression of apoptotic-related proteins caspase 3 and Bax, lower expression of antiapoptotic-
related protein Bcl-2 compared to control groups. Also, co-treatment with kaempferol lowered the expression of caspase 3 and Bax/Bcl-2 compared to the HFD-OVX group (Figure 7(C–E)).

**Effects of kaempferol on the ROS accumulation and the level of inflammatory factors in HAECs treated with ox-LDL**

Following the assessment of the effects of kaempferol on oxidative stress and inflammatory factors in vitro, we found that ox-LDL-induced intracellular ROS accumulation was significantly suppressed after kaempferol co-treatment (Figure 8(A)). Similarly, ox-LDL elevated the intracellular concentrations of IL-6 and TNF-α, whereas kaempferol administration obviously decreased the level of inflammatory factors (Figure 8(E,F)). Further evaluation of the expression of intercellular and vascular adhesion molecules (ICAM and VCAM) through western blotting indicated that ox-LDL-induced expression of ICAM and VCAM was higher, more than twofold, compared to the control group. On the contrary, kaempferol, at concentrations of 5, 10 and 20 μM, lowered the expression of ICAM and VCAM. Notably, following treatment with 20 μM kaempferol, the expression of ICAM and VCAM recovered to an approximately normal level compared to the control group (Figure 8(B–D)). Therefore, it was deduced that kaempferol attenuated oxidative stress and blocked the release of inflammatory factors in HAECs.

**Effects of kaempferol treatment on GPER expression and PI3K/AKT/Nrf2 pathway in HAECs treated with ox-LDL**

After treatment, HAECs were collected to detect the protein expression of GPER, PI3K, P-AKT/AKT, Nrf2 and HO-1. Compared to the control group, GPER protein expression was significantly lower after ox-LDL administration, whereas kaempferol increased GPER expression (Figure 9(A)). Besides, ox-LDL lowered the expression of PI3K, HO-1, Nrf2 and the ratio of p-AKT/AKT. Similarly, kaempferol treatment elevated the expression of PI3K, HO-1, Nrf2 and the ratio of p-AKT/AKT (Figure 9(B–F)). These results implied that kaempferol induced the activation of GPER and upregulation of the PI3K/AKT/Nrf2 pathway.

**GPER activation is associated with the protective effect of kaempferol against AS**

In this experiment, we evaluated the potential role of GPER in the protective effect of kaempferol against AS via a transient transfection experiment which demonstrated rare TUNEL-positive cells in the control group but more TUNEL-positive cells in the ox-LDL group. Thus, it was evident that ox-LDL induced apoptosis in HAECs (Figure 10(A)). However, kaempferol treatment notably inhibited ox-LDL-induced apoptosis, which was largely reversed after transfection of si-GPER. This contributed to the release of more apoptotic cells. In parallel to the apoptotic assay, the inhibition of kaempferol in the ROS generation diminished after si-GPER transfection (Figure 10(B)). Also, we explored the effect of si-GPER on the protein expression of PI3K, Nrf-2 and P-AKT. GPER, PI3K, Nrf-2 and the ratio of p-AKT/AKT expression were markedly decreased in HAECs exposed to ox-LDL, whereas kaempferol (20 μM) increased the protein expression of GPER, PI3K, Nrf-2 and ratio of p-AKT/AKT. However, the modulation of kaempferol on protein expression of GPER, PI3K, Nrf-2 and the ratio of p-AKT/AKT was significantly abolished after si-GPER transfection (Figure 10(C–G)). These findings suggested that kaempferol-induced GPER activation potentially regulates the PI3K/AKT/Nrf2 pathway, consequently alleviating AS.

**Discussion**

Atherosclerosis gradually became the main factor attributed to the high incidence and mortality in developed and developing countries. For a long time, many research groups and scholars have dedicated their efforts towards curbing this disease (Fredman and Tabas 2017). In most cases, animal models are employed to explore the aetiology, pathological changes in AS, and the beneficial effects of anti-atherosclerotic medicine (Lee et al. 2017). Recent studies have demonstrated that
Figure 8. Kaempferol inhibited the ROS accumulation and reduced the level of inflammation factors. HAECs cells were treated with kaempferol or free-foetal medium. Then, the cells were treated with indicated drugs or complete medium. The changes in ROS accumulation (A), the expression of ICAM-1 (C) and VCAM-1 (D) and level of TNF-α (E) and IL-6 (F) in HAEC cells were shown. Data are analysed by one-way ANOVA and unpaired t-test and presented as the mean ± SD. ##p < 0.01 and ###p < 0.001 vs. the control group. *p < 0.05 and **p < 0.01 and ***p < 0.001 vs. the ox-LDL group. ns: not significant (p > 0.05 vs. the control group) (n = 5).

Figure 9. Kaempferol activated the GPER and regulated the PI3K/AKT/Nrf2 Pathway. HAECs cells were treated with kaempferol or free-foetal medium. Then, the cells were treated with indicated drugs or complete medium. The expression of GPER (A), PI3K (C), P-AKT/AKT (D), Nrf2 (E) and HO-1 (F) was detected by Westernblot assay. Data are analysed by one-way ANOVA and unpaired t-test and presented as the mean ± SD. ##p < 0.01 vs. the control group. *p < 0.05 and **p < 0.01 vs. the ox-LDL group. ns: not significant (p > 0.05 vs. the control group) (n = 5).
Postmenopausal women are associated with a higher risk of AS than premenopausal women, implying the potential association of oestrogen with AS (Huang et al. 2020). For instance, a study found that female mice who underwent OVX were more susceptible to AS than normal mice (Jing et al. 2017). In the present study, by evaluating the potential cardiovascular effects of kaempferol in HFD-OVX induced APOE−/− mice, we found more severe vascular lesions in the OVX-HFD group compared to normal mice. This indicated that a successful model of postmenopausal AS was achieved in mice. Additionally, kaempferol treatment alleviated pathological aortic changes in OVX-HFD induced APOE−/− mice.

Compelling evidence has confirmed that oestrogen plays a core role in the cardiovascular system by regulating the vascular physiological and pathological conditions, implicating oestrogen as a potential cardioprotective agent (Dworatzek and Mahmoodzadeh 2017). However, many side effects have been reported in patients treated with oestrogen replacement therapy (Schierbeck 2015). Contrarily, plant-derived oestrogen is associated with minimal side effects, therefore, has a broad application prospect. The effect of oestrogen on cardiovascular function is mediated by nuclear and membrane ERs, including oestrogen receptor α (ERα), oestrogen receptor β (ERβ) and G protein-coupled ER (GPR30 or GPER) (Han et al. 2013). Recent evidence found that GPER knockout aggravated the progression of AS in ovariectomized mice, whereas GPER agonist-treatment alleviated postmenopausal AS in ovariectomized mice (Yang et al. 2017). Herein, we found low expression of GPER and severe AS was reported in the OVX group. Besides, kaempferol treatment elevated GPER expression and alleviated vascular lesions. Recent study suggested that oestrogen loss was associated with lipid levels, inflammation and apoptosis in AS (Yang et al. 2019). In the present study, OVX-HFD was associated with high LDL-C, TG, T-CHO levels and low HDL-C level; however, these changes were reversed following kaempferol treatment. Moreover, OVX-HFD induced elevated oxidative stress level, release of inflammatory factors and apoptosis. Similarly, kaempferol reduced the over-generation of ROS, level of inflammation and apoptosis. Based on these observations, we concluded that kaempferol-induced GPER activation potentially inhibit inflammation, oxidative stress and apoptosis to protect against AS.

PI3K/AKT/Nrf2 signalling pathway is associated with inflammation, oxidative stress, free radical release and apoptosis (Chen et al. 2019; Xiong et al. 2020). Several studies have reported that activation of this pathway alleviates the injury in numerous organs, including the retina, lung, liver, kidney, etc. (Reddy et al. 2015; Zhang G et al. 2016; Xu et al. 2017; Thangapandiyan et al. 2019). Also, there is a close relationship between the PI3K/AKT/Nrf2 signalling pathway and the level of oestrogen; this may impact the progression of AS (Hwang and Jeong 2010). Similarly, Gu et al. found that the upregulation of PI3K could attenuate the damage of endothelial cells and inhibit AS progression in APOE mice (Gu et al. 2019). Therefore, the PI3K/AKT/Nrf2 signalling pathway potentially exerts beneficial effects in AS. We found that kaempferol is associated with high expression of PI3K, AKT, Nrf2 and low level of inflammatory factors, which
consequently alleviate AS-induced injury. However, the correlation between GPER expression and PI3K/AKT/Nrf2 signalling pathway remains elusive and warrants further exploration.

Numerous reports revealed that loss of oestrogen induces the activation of oxidative stress and increases inflammation and apoptosis. ox-LDL is an essential factor in AS. It acts on endothelial cells, macrophages, platelets, fibroblasts and smooth muscle cells, aggravating the formation and vulnerability of atherosclerotic plaques and eventually contributes to inflammation and apoptosis (Kattoor et al. 2019). Chang et al. administrated ox-LDL, an agent for establishing the model, to endothelial cells and macrophages to investigate the mechanism of AS in vitro (Chang et al. 2019). Our present results showed that the IC50 of ox-LDL to HAECS cells was approximately 200 μg/mL (Figure 6(A)), whereas kaempferol exerted no toxicity effects to HAECS cells below 40 μM (Figure 6(B)). On this basis, ox-LDL 200 μg/mL and kaempferol were co-treated in HAECS to explore the protective effects of kaempferol on AS. The results demonstrated that kaempferol increased cell viability and alleviated ox-LDL-induced apoptosis and inflammation. Therefore, we validated the in vitro protective effect of kaempferol on AS. Moreover, the GPER gene knockout experiment was carried out to further explore the mechanism of kaempferol protecting against AS and the relationship between GPER and PI3K/AKT/Nrf2 signal pathway. After the knockdown of GPER, the inhibiting effects of kaempferol on oxidative stress, inflammation and apoptosis were significantly weakened, and the PI3K/AKT/Nrf2 signal pathway was down-regulated (Figure 10). These results suggest that kaempferol-induced GPER upregulation attenuated AS by modulating the PI3K/AKT/Nrf2 pathway to inhibit oxidative stress, inflammation and apoptosis.

For a long time, plant-derived oestrogen has been adopted as a therapeutic agent for human diseases, and currently, it plays an important role in health care practice. Recent findings indicate that the administration of phytoestrogens may be beneficial for the management of cancer, osteoporosis and neurodegenerative diseases (Lagarì and Levis 2013; Langasco et al. 2019; Peiffer et al. 2020). With the advancement in science and more in-depth research, researchers extracted and separated effective phytoestrogens from Chinese herbal medicine and transformed the traditional medicine treatment to elucidate the underlying molecular mechanism (Wang X et al. 2006). Attractively, numerous studies found that phytoestrogens present satisfactory results as potential therapeutic options for AS (Kirichenko et al. 2017). In our current study, kaempferol alleviated OVX-HFD-derived AS in vivo and ox-LDL-induced injury in vitro. However, there are many obstacles to overcome a successful translation from basic experiments to clinical application. Future experiments should be conducted to explore the mechanism of kaempferol in alleviating AS to bring prosperity for its prevention and treatment, especially postmenopausal AS.

Conclusions

The present work elucidated the protective effects of kaempferol on postmenopausal AS. It uncovers a new foresight into the further molecular mechanisms of kaempferol against postmenopausal AS. Of note, in ovariectomized mice treated with HFD, kaempferol pre-administration improved the morphology of blood vessels, normalized the lipid levels, and suppressed the level of ROS over-generation, inflammation and apoptosis significantly. Kaempferol was associated with high GPER expression and upregulation of the PI3K/AKT/Nrf2 signal pathway. Besides, the assay of GPER specific siRNA transfection indicated that the protective effect of kaempferol on postmenopausal AS is involved in activating the PI3K/AKT/Nrf2 pathway via the activation of GPER.

Author contributions

Huijun Sun conceived and designed this study, Zhuo Feng conducted the experiments, collected the data and drafted the manuscript. Yue, Jin, Changyuan, Wang, Qiang Meng and Jingjing Wu provided technological support and contributed to other works. All the authors read and approved this manuscript.

Disclosure statement

All authors declare that there are no conflicts of interest.

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ORCID

Huijun Sun http://orcid.org/0000-0003-4474-6966

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

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