Homoplantaginin Modulates Insulin Sensitivity in Endothelial Cells by Inhibiting Inflammation

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Recent data have indicated that inflammation plays an important role in the development of insulin resistance. The present study aims at examining the activity of homoplantaginin, a flavonoid from a traditional Chinese medicine *Salvia plebeia* R. Br., on palmitic acid (PA)-induced insulin sensitivity and the underlying mechanisms of its anti-inflammatory properties in the endothelial cells. Pre-treatment of homoplantaginin on human umbilical vein endothelial cells (HUVECs) significantly inhibited PA induced tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) mRNA expression, and inhibitory κ B kinase beta (IKK β) and nuclear factor- κ B (NF- κ B) p65 phosphorylation. To the PA-impaired insulin-dependent tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and decrease in nitric oxide (NO) production, pretreatment of homoplantaginin could effectively reverse the effects of PA. Additionally, homoplantaginin significantly modulated the Ser/Thr phosphorylation of IRS-1, improved phosphorylation of Akt and endothelial nitric oxide synthase (eNOS), and increased NO production in the presence of insulin. Taken together, our results demonstrated that homoplantaginin ameliorates endothelial insulin resistance by inhibiting inflammation and modulating cell signalling via the IKK β /IRS-1/pAkt/eNOS pathway, suggesting it may be used for the prevention and treatment of endothelial dysfunction associated with insulin resistance.

Key words homoplantaginin; inflammation; insulin resistance; endothelial cell

Insulin resistance is one of the defining clinical features of metabolic syndrome (MetS) and is especially characteristic of most patients with type 2 diabetes mellitus (T2DM). Endothelial dysfunction is widespread among type 2 diabetes patients and individuals with insulin-resistant conditions.¹⁾ Endothelial dysfunction is a hallmark of diabetic vascular disease and can be described as an impairment of the generation and function of nitric oxide (NO), a vasodilator and vascular homeostatic agent.²⁾ One of the physiological actions of insulin in the vasculature is to promote vasodilation by increasing NO production. In endothelial cells, insulin increases NO production through an insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-kinase)-dependent pathway³⁾ that mediates the phosphorylation of endothelial nitric oxide synthase (eNOS) by Akt in a calcium-independent manner.⁴⁾

In recent years, low grade inflammation has been identified as one of the factors associated with endothelial dysfunction in subjects with type 2 diabetes,¹⁾ with free fatty acids (FFAs) representing one class of inflammatory factors involved in the process.⁵⁾ It has been reported that the plasma free fatty acid elevation is closely related to the development of insulin resistance.⁶⁾ FFAs have also been shown to activate inhibitory κ B kinase beta (IKK β), a serine kinase that controls the activation of the inflammation-associated nuclear factor- κ B (NF- κ B) transcription factor. FFAs increase IRS-1 serine phosphorylation in cultured endothelial cells, which subsequently reduces insulin-dependent IRS-1 tyrosine phosphorylation, decreasing the activation of PI3-kinase and NO production.⁷⁾

Homoplantaginin is believed to be the main flavonoid component in *Salvia plebeia* R. Br. (Labiatae),⁸⁾ a traditional Chinese medicine used for the treatment of a variety of inflammatory diseases including hepatitis, cough, diarrhea, gonorrhea, menorrhagia, tumors and hemorrhoids.⁹⁾ *Salvia plebeia* R. Br. was identified to be a potent antioxidant plant.¹⁰⁾ The compound homoplantaginin in *Salvia plebeia* could ameliorate

liver injury. Homoplantaginin markedly decreased the levels of tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) in Bacillus Calmette–Guérin/lipopolysaccharide-induced hepatic injury mice.¹¹⁾ Our previous study revealed that homoplantaginin could significantly attenuate hyperlipidemia and hyperglycemia in diabetic mice.¹²⁾ However, its anti-inflammatory potency in the endothelium, particularly in the context of endothelial insulin resistance, has not been reported. In this study, we investigated the effect of homoplantaginin on FFA-induced insulin resistance in endothelial cells and to better understand the relative mechanism underlying its regulation of insulin signaling transduction.

MATERIALS AND METHODS

Reagents Palmitate acid (PA, Sinopharm Chemical Reagent Co., Ltd., China) was dissolved in ethanol at 200 mM as stock solution and further diluted to 5 mM with 10% FFA-free and low endotoxin bovine serum albumin before the use. Sodium salicylate was purchased from Tianjin Kemiou Chemical Agent Center; (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and insulin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Phospho-IRS-1 (Ser307), IRS-1(R301) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibodies were purchased from Bioworld Technology, MN, U.S.A. Phospho-Akt (T308), Akt (A444), phospho-eNOS (Ser1177), phospho-NF- κ B p65 (Ser536), NF- κ B p65, phospho-IKK β (Y199), and IKK β (F182) monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.).

Isolation and Purification of Homoplantaginin Powdered herbs of *Salvia plebeia* R. Br. were refluxed with 85% ethanol three times. The extract was suspended in water and successively partitioned with petroleum ether, ethyl acetate (EtOAc). The EtOAc fraction was separated by silica gel

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column chromatography using a gradient of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (100:1→1:1), then $\text{CHCl}_3/\text{CH}_3\text{OH}$ (100:8) fraction was subjected to Sephadex LH-20 column chromatography to obtain homoplantagin. The chemical structure was identified through spectroscopic analysis as reported previously.¹³ The purity of this compound was determined to be higher than 98% by normalization of the peak area detected by high-performance liquid chromatography (HPLC). Homoplantagin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, stored at -20°C , and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% DMSO throughout the study (control groups were always treated with 0.1% DMSO in the corresponding experiments).

Cell Culture The human umbilical vein endothelial cells (HUVECs) were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2 and 95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin. When cells grew to 80% confluence, the culture medium was replaced, and the cells were starved for 24 h in serum-free medium prior to PA treatment.

Cell Viability Assay The viability of cultured cells was determined using the reduction of MTT to formazan. HUVECs (1×10^4 cells/well) were plated in 96-well plates and incubated at 37°C in a 5% CO_2 incubator. The cells were then exposed to various concentrations of homoplantagin (0.1, 1, 3, 10, 30, 100 μM) for 48 h. Subsequently, 20 μL of MTT (5 mg/mL) was added to each well for an additional 4 h at 37°C . The supernatant was removed, and DMSO was added to dissolve the formazan crystals. The optical absorbance was measured at 540 nm.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) IL-6 and TNF- α mRNA levels in HUVECs were measured by RT-PCR method. Briefly, 1×10^6 cells were cultured in DMEM (10% fetal calf serum) for 24 h. The cells were then preincubated with homoplantagin (0.1, 1 or 10 μM) or sodium salicylate (500 μM) for 30 min, followed by stimulation with PA (100 μM). After 3 h incubation with PA, total RNA in the cells was extracted using TriZol Reagent (Invitrogen, U.S.A.). 5 μg of total RNA was reverse-transcribed into cDNA using an EasyScript First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech). The individual target cDNA was amplified using specific primers as follows: 5'-TAGCGGCCTCACACAGACAG-3' (sense) and 5'-GGC TGGCATATGTGCTTGGG-3' (antisense) for IL-6 (408 bp); 5'-CTCTGGCCGAGGCAGTCAGA-3' (sense) and 5'-GGC GTTTGGGAAGGTTGGAT-3' (antisense) for TNF- α (519 bp); and 5'-ACATCTGCTGGAAGGTGGAC-3' (sense) and 5'-GGT ACCACCATGTACCCAGG-3' (antisense) for β -actin (161 bp). PCR products were subjected to electrophoresis in 1.5% agarose gels with 0.5 $\mu\text{g/mL}$ ethidium bromide and visualised under UV light; the signal intensity was then measured using a Bio-Rad image system and analysed using Quantity One software (Bio-Rad). The mRNA levels of individual genes were normalised and represented as ratios against β -actin mRNA levels.

Western Blot Analysis After being treated with PA or insulin, HUVECs were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffer (1%

TritonX-100, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 $\mu\text{g/mL}$ leupeptin, 20 $\mu\text{g/mL}$ aprotinin, 125 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium orthovanadate). The cell lysates were achieved by centrifugation at 12,000 rpm for 10 min at 4°C . Equivalent amounts of protein determined by a Bradford assay were separated in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking in a solution (5% skimmed milk in Tris-buffered saline (TBS) containing 0.05% Tween 20), the membranes were incubated with primary antibody (1:1000 dilution) at 4°C overnight. Then membranes were washed and reacted with horseradish peroxidase (HRP)-labeled anti-rabbit immunoglobulin G (1:2000 dilution) for 1 h at 37°C . Membranes were incubated with an enhanced chemical luminescence detection solution and exposed to X-ray film. The resulting images were analyzed with Image J software (National Institutes of Health, U.S.A.).

Measurement of Intracellular NO Production The NO-specific fluorescent dye 4-amino, 5-aminomethyl-2',7'-difluorescein, diacetate (DAF-FM DA) was used to assess NO production in endothelial cells as described previously.¹⁴ Briefly, HUVECs (1×10^4 cells/well) were incubated with homoplantagin (0.1 or 1 μM) or sodium salicylate (500 μM) for 30 min, followed by stimulation with PA (100 μM) for 3 h. After being washed with cold PBS, the cells were treated with 5 μM DAF-FM DA for 30 min at 37°C . Subsequently, residual extracellular chromophore was washed away with ice-cold PBS and insulin (100 nM) or diluent was added for 5 min. The cells were then fixed with 4% paraformaldehyde for 5 min at 4°C . The fluorescence in fixed cells was measured using an Olympus fluorescence microscope (Olympus, IX51S8F-3) with excitation and emission wavelengths of 495 and of 515 nm, respectively. All images were taken in the first 30 s of light exposure to avoid fluorescence decay. Fluorescence intensity of the images was analyzed with Image J software (National Institutes of Health, U.S.A.).

Statistic Analysis All data are expressed as mean $\pm$ S.D. from three or four independent experiments. Statistical differences were evaluated by the one-way analysis of variance (ANOVA) and two-tailed Student's *t*-test. A $p < 0.05$ was considered significant.

RESULTS

Effect of Homoplantagin on the Viability of HUVECs HUVECs were cultured with various concentrations of homoplantagin for 48 h, and cell viability was evaluated using the MTT assay. As shown in Fig. 1, treatment with homoplantagin (0.1, 1, 3, 10, 30 and 100 μM), the cell viability was 103%, 95%, 99%, 100%, 101% 99% of the blank, respectively. These results demonstrated that homoplantagin did not exhibit substantial cytotoxicity on HUVECs at concentrations up to 100 μM .

Effects of Homoplantagin on mRNA Expressions of Proinflammatory Cytokines and Activation of IKK β /NF- κB in PA-Treated HUVECs To determine its anti-inflammatory activity, we first examined the effects of homoplantagin on IL-6 and TNF- α mRNA expression in PA-treated HUVECs. As shown in Fig. 2, the IL-6 and TNF- α mRNA levels in PA (100 μM) treated HUVECs were significantly

Fig. 1. Effect of Homoplantagin on the Viability of HUVECs

Cells were cultured with various concentrations of homoplantagin for 48 h in 96-well plates. Cell viability was assessed using the MTT assay. The data shown are expressed as the mean values $\pm$ S.D. of three independent experiments.

increased by 2.7-fold and 1.7-fold, respectively, when compared to blank. Pretreatment of homoplantagin suppressed IL-6 mRNA expression by 20%, 35% and 56% and TNF- α mRNA expression by 46%, 54% and 80% at 0.1, 1 and 10 μ M, respectively. The positive control sodium salicylate showed a similar inhibitory effect on TNF- α and IL-6 mRNA expression.

IKK β is a serine kinase that controls the activation of NF- κ B, a transcription factor associated with inflammation, and can be activated by PA, one of the richest FFAs. As shown in Fig. 3, PA significantly increased the phosphorylation of IKK β and NF- κ B, by 1.8-fold and 4.7-fold, respectively, compared to the blank. Interestingly, pre-treatment with homoplantagin significantly decreased PA-induced IKK β and NF- κ B phosphorylation in a dose dependent manner. Pretreatment of homoplantagin suppressed IKK β phosphorylation by 95%, 106% and 162% and NF- κ B phosphorylation by 57%, 86% and 102% at 0.1, 1 and 10 μ M, respectively. Likewise, sodium salicylate also inhibited IKK β and NF- κ B activation evoked by PA. These results suggest that homoplantagin has significant anti-inflammatory activity on HUVECs.

Effect of Homoplantagin on Serine Phosphorylation of IRS-1 in PA-Stimulated HUVECs To determine whether its anti-inflammatory potency could contribute to an inflammation-mediated enhancement of insulin resistance, we examined the effect of homoplantagin on serine phosphorylation of IRS-1 in PA-stimulated HUVECs. As shown in Fig. 4, when endothelial cells were exposed to PA, the phosphorylation level of serine residue S307 in IRS-1 was increased by 2.1 fold compared to blank. When the cells were pretreated with homoplantagin, the phosphorylation of IRS-1 S307 residue was reduced, and the inhibition was about 12%, 46% and 55% at 0.1, 1 and 10 μ M, respectively. Similarly, sodium salicylate decreased the phosphorylation of IRS-1 and the inhibition ratio is 29%.

Effects of Homoplantagin on the Tyrosine Phosphorylation of IRS-1 and Phosphorylation of Akt, eNOS in HUVECs Induced by PA To further demonstrate that the anti-inflammatory potency of homoplantagin contributed to amelioration of insulin resistance, we examined its effect on the phosphorylation of tyrosine in IRS-1 (detected by Western blot analysis using the PY99 antibody) in PA-induced

Fig. 2. Effect of Homoplantagin on IL-6 and TNF- α mRNA Expression in PA-Treated HUVECs

HUVECs were pretreated with homoplantagin or sodium salicylate for 30 min before being incubated with PA for 3 h. mRNA expression levels were determined using RT-PCR. Salicylate-treated cells were used as positive controls. The data shown are expressed as the mean values $\pm$ S.D. of three independent experiments. $^{\#}p < 0.05$, $^{##}p < 0.01$ versus blank; $^*p < 0.05$ versus control.

endothelial cells. As shown in Fig. 5A, following PA treatment, the insulin-mediated tyrosine phosphorylation of IRS-1 was significantly reduced. Pretreatment with homoplantagin (0.1, 1, 10 μ M) remarkably increased the level of the IRS-1 tyrosine phosphorylation, by 2.2, 2.3 and 2.6-fold, respectively, compared to control. Additionally, PA treatment significantly impaired this insulin-mediated tyrosine phosphorylation in Akt and eNOS. Pretreatment with homoplantagin (0.1, 1, 10 μ M) remarkably increased Akt phosphorylation by 1.3, 2.4 and 2.8-fold (Fig. 5B), and eNOS phosphorylation by 1.1, 1.6 and 1.9-fold (Fig. 5C), respectively.

Effect of Homoplantagin on NO Production in PA-Treated HUVECs Under normal circumstances, insulin-stimulated Akt phosphorylation can activate eNOS to produce NO. Intracellular NO production was detected as an increase in fluorescence intensity in loaded HUVECs cells. Representative fluorescent images of DAF-FM DA-loaded cells are shown in Fig. 6A. As a result, 100 nM of insulin induced a significant increase in NO production, while 100 μ M of PA strongly abolished insulin-induced increases in NO production (Fig. 6B). In contrast, pretreatment with homoplantagin or

Fig. 3. Effect of Homoplantagin on IKK β /NF- κ B Activation in PA-Treated HUVECs

HUVECs were pretreated with homoplantagin or salicylate before being stimulated with PA (100 μ M) for 3 h. "Blank" and "control" indicate the absence or presence of PA, respectively. IKK β and p65 protein phosphorylation was assessed using Western blot analysis. The data shown are expressed as the mean values $\pm$ S.D. of four independent experiments. [#] p < 0.05 versus blank; ^{*} p < 0.05, ^{**} p < 0.01 versus control.

sodium salicylate significantly blocked PA-induced decreases in NO production (Fig. 6B). Compared to control, NO production was increased by up to 54% and 45% with 0.1, 1 μ M homoplantagin, respectively.

DISCUSSION

The insulin resistance syndrome refers to a constellation of findings, including glucose intolerance, obesity, dyslipidemia, and hypertension, that promote the development of type 2 diabetes, cardiovascular disease, cancer, and other disorders.¹⁵ Insulin resistance is a central component of metabolic syndrome and an important pathophysiological factor in the development of type 2 diabetes as well as cardiovascular diseases.¹⁶ Endothelial dysfunction is commonly observed in association with type 2 diabetes and other situations characterised by insulin resistance.¹⁷⁻¹⁹ Insulin resistance and endothelial dysfunction may co-exist, where chronic inflammation may be a crucial factor. The early onset of vascular inflammation was accompanied by biochemical evidence of both endothelial

Fig. 4. Effect of Homoplantagin on Serine Phosphorylation of IRS-1 in PA-Stimulated HUVECs

HUVECs were pretreated with homoplantagin or salicylate for 30 min before being incubated with 100 μ M PA for 3 h. "Blank" and "control" indicate the absence or presence of PA, respectively. Protein phosphorylation levels were determined using Western blot analysis. The bar graphs show p-IRS (S307) levels as a percentage of IRS expression. The data shown are expressed as the mean values $\pm$ S.D. of three independent experiments. ^{##} p < 0.01 versus blank; ^{**} p < 0.01 versus control.

dysfunction (*i.e.* reduced nitric oxide production, induction of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1) and insulin resistance (impaired insulin-induced phosphorylation of Akt and eNOS).²⁰

Previous study related to the interplay between obesity, inflammation and insulin resistance revealed that TNF expression was elevated in adipose tissues isolated from multiple experimental models of obesity.^{21,22} Subsequently, similar correlations between TNF levels, obesity and insulin resistance in humans were suggested.²³ TNF- α , as one of the proinflammatory cytokines, was shown to interrupt the early insulin-stimulated tyrosine phosphorylation events, which is crucial to insulin transmembrane signaling.²⁴ Interleukin (IL)-6 is released in substantial amounts not only by monocytes and hepatic Kupffer cells, but also by endothelial cells, skeletal muscle cells, and adipocytes.²⁵ In addition, IL-6 is another inflammatory cytokines known to be elevated in obesity and correlated with insulin resistance.^{26,27} Therefore, TNF- α and IL-6 seems to be crucial player in the metabolic syndrome.

Obesity-linked insulin resistance is associated with chronic inflammation and cardiovascular complications. Free fatty acids (FFAs) are prominent candidates for the molecular link between these disorders. High levels of plasma FFAs impair endothelial function and are associated with a number of cardiovascular risk factors linked to insulin resistance, including hypertension, dyslipidemia, and abnormal fibrinolysis.⁷ FFAs could induce inflammatory response and insulin resistance in macrophages, adipocytes and endothelial cells through the innate immune receptor Toll-like receptor-4 (TLR4).^{28,29} In this context, PA, one of the richest FFAs in the plasma of obesity patients, was chosen as a pathogenic agent to induce inflammatory response and insulin resistance in cultured endothelial cells. Our results showed that, IL-6 and TNF- α mRNA levels significantly increased in HUVECs exposed to PA (100 μ M). Pretreatment of homoplantagin concentration-dependently suppressed IL-6 and TNF- α mRNA expression. These results

Fig. 5. Effects of Homoplantagin on the Tyrosine Phosphorylation of IRS-1 and Phosphorylation of Akt, eNOS in HUVECs

HUVECs were incubated with PA (100 μ M) for 3 h before being treated with insulin (100 nM) for 15 min. Cells were pretreated with either homoplantagin or salicylate for 30 min prior to treatment with PA. Protein phosphorylation levels were determined using western blot analysis. The bar graphs show PY99-IRS levels as a percentage of IRS expression. The bar graphs also show p-Akt levels as a percentage of Akt expression and p-eNOS levels as a percentage of eNOS expression. The data are expressed as the mean values $\pm$ S.D. of four independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus blank 2; $^*p < 0.05$, $^{**}p < 0.01$ versus control.

indicated that homoplantagin inhibited vascular inflammation by down-regulation of TNF- α and IL-6 gene expression (Fig. 2).

IKK is made up of two kinases, IKK α and IKK β . IKK β is the primary subunit of the IKK complex involved in mediating the proinflammatory signal-induced phosphorylation of NF- κ B inhibitory protein (I κ B α), which leads to the degradation of I κ B α and the consequent activation of NF- κ B.^{30,31} NF- κ B is a critical mediator of inflammation progress and regulates the expression of a wide range of inflammatory molecules such as inducible nitric oxide synthase, cyclooxygenase-2, IL-6 and TNF- α .³² In the present study, PA induced IKK β activation in endothelial cells, which subsequently promoted the activation of NF- κ B, a transcription factor associated with inflammation. Moreover, endothelial cells treated with homoplantagin, the phosphorylation of IKK β was significantly reduced and the activation of NF- κ B was remarkably inhibited (Fig. 3). These results suggested that homoplantagin inhibited PA induced TNF- α and IL-6 gene expression by an IKK β /NF- κ B-dependent pathway in endothelial cells.

Many evidences have showed that activation or overexpression of IKK β will attenuate the insulin signalling. Salicylate acting as inhibitors of IKK- β and NF- κ B, could be used to treat insulin resistance and type 2 diabetes.³³ At the molecular level, inhibition of IKK β prevented the Ser/Thr

phosphorylation of IRS-1, whereas it improved the insulin-stimulated Tyr phosphorylation of IRS-1, thus implicating IKK β as the IRS kinase.³⁴ Recent data suggest that IRS-1 may be directly phosphorylated by IKK β at serine residues.⁷ Phosphorylation of the serine residue of IRS-1 blocks the interaction between the tyrosine residue of IRS-1 and the insulin receptor. This subsequently affects the PI3K/Akt pathway, in turn reducing endothelial eNOS transcription and NO generation and ultimately resulting in insulin resistance.^{4,21} These findings may represent a mechanism by which the inflammatory pathways contribute to impaired insulin signaling.

In the current study, we further investigated whether homoplantagin could ameliorate insulin sensitivity *via* its inhibition of IRS-1 (Ser307) phosphorylation. Our results showed that pretreatment with homoplantagin and sodium salicylate reduced the PA-induced phosphorylation of the IRS-1 serine residue (Ser307) (Fig. 4). The inhibition of homoplantagin on NF- κ B-dependent inflammation might be responsible for the PA induced down-regulation of IRS-1 serine phosphorylation.

Endothelial-derived NO plays a key role in maintaining normal endothelial function, which includes inhibition of thrombosis and vascular inflammation and maintaining blood pressure and vessel patency. NO also inhibits abnormal growth and inflammation, exerts antiaggregatory effects on

Fig. 6. Effect of Homoplantagin on NO Production in PA-Treated HUVECs

Photographs are representative fluorescent images of HUVECs treated with DAF-FM DA. The images shown were captured 5 min after the cells were stimulated with insulin. A: a, Blank 1, Image of cells with no drug treatment; b, Blank 2, Image of cells with cultured with 100 nM insulin; c, control; d, 0.1 μM homoplantagin; e, 1 μM homoplantagin; f, 500 μM Salicylate. Magnification: ×200. B The bar graphs show fluorescence intensity represented NO production in endothelial cells. The data shown are expressed as the mean values ± S.D. of three independent experiments. # $p < 0.05$, ## $p < 0.01$ versus blank 2; * $p < 0.05$, ** $p < 0.01$ versus control.

platelets, and promotes vasodilation. Previous work has demonstrated that the IRS-1/PI3-kinase/pAkt/peNOS pathway is important for endothelial NO production. Transfection with mutated IRS-1, inhibitory PI3-kinase mutants, or dominant-negative Akt abrogate insulin dependent NO production.⁷⁾ Many of the metabolic abnormalities found in diabetes and obesity, which include elevation of TNF- α and metabolites such as FFAs, have been shown to have negative effects on the IRS-1/PI3-kinase signaling pathway in myocytes and hepatocytes. Our data indicated that PA impaired insulin-dependent NO production through downregulation of cell signaling via the IRS-1/pAkt/peNOS pathway. As expected, we found that homoplantagin inhibited IRS-1 serine phosphorylation of (Ser307), subsequently enhancing IRS-1 Tyr phosphorylation (PY99) (Fig. 5A), PI3-kinase activation (T308) (Fig. 5B), eNOS phosphorylation (Fig. 5C), and, ultimately improved NO production (Fig. 6).

In summary, the present study demonstrates that homoplantagin ameliorates endothelial insulin resistance by inhibiting inflammation and modulating cell signalling via the IKK β /

IRS-1/pAkt/peNOS pathway. Previously, we have demonstrated that homoplantagin could attenuate hyperlipidemia and hyperglycemia in diabetic mice. Thus, homoplantagin may potentially be used for the prevention and treatment of cardiovascular disease associated with insulin resistance and diabetes.

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