**Baicalin, baicalein and wogonin inhibits high glucose-induced vascular inflammation in vitro and in vivo**

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Running title: Effect of baicalin, baicalein and wogonin on diabetes

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Supplementary Materials and methods

Reagents

Compounds (baicalin, baicalein and wogonin), D-glucose, L-glucose, D-mannitol, Evans blue, 2-mercaptoethanol, and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as described previously (1). Briefly, cells were cultured to confluency at 37°C and 5% CO₂ in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science). THP-1 cells, a monocyte cell line, were maintained as previously described (2).

Animals and husbandry

Male C57BL/6 mice (6-7-wk old, weighting 18-20 g) purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea) were used in this study after a 12-day acclimatization period. Animals were housed five per polycarbonate cage under controlled temperature (20-25°C) and humidity (40% - 45%) and a 12:12 hour light/dark cycle. Animals were supplied a normal rodent pellet diet and water ad libitum during acclimatization. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University (KNU2012-13).
**Cell viability assay**

MTT was used as an indicator of cell viability. Cells were grown in 96-well plates at a density of 5 x 10^3 cells/well. After 24 h, cells were washed with fresh medium, followed by treatment with each compound. After a 48 h incubation period, cells were washed, and 100 µl of MTT (1 mg/ml) was added, followed by incubation for 4 h. Finally, DMSO (150 µl) was added in order to solubilize the formazan salt formed and the amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (Tecan Austria GmbH, Austria).

**Permeability assay in vitro**

Endothelial cell permeability in response to increasing concentrations of each compound was quantified by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional cell monolayers using a modified 2-compartment chamber model as previously described (3). HUVECs were plated (5 x 10^4/well) in 3-µm pore size, 12-mm diameter transwells for three days. Confluent monolayers were incubated with increasing concentrations of each compound for 6 h followed by incubation with indicated concentrations of high glucose for 24 h. Then, Transwell inserts were washed with PBS (pH 7.4), followed by addition of 0.5 ml of Evans blue (0.67 mg/ml) diluted in growth medium containing 4% BSA. Fresh growth medium was then added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. Ten minutes later, optical density was measured at 650 nm in the lower chamber.

*In vivo permeability and the leukocyte migration assay*
Mice were pretreated with intravenous administration of each compound, and, after 6 h, 1% Evans blue dye solution in normal saline was administered by intravenous injection in each mouse immediately followed by an intravenous injection of high glucose (9 mg/kg). Thirty minutes later, the mice were sacrificed and the peritoneal exudates were collected after being washed with 5 ml of normal saline and centrifuged at 200 xg for 10 min. The absorbance of the supernatant was read at 650 nm. Vascular permeability was expressed in terms of dye (µg/mouse), which leaked into the peritoneal cavity according to a standard curve of Evans blue dye, as previously described (4-5).

For assessment of leukocyte migration, mice were euthanized, and peritoneal cavities were washed with 5 mL of normal saline. Samples (20 µl) of peritoneal fluids were mixed with 0.38 mL of Turk's solution (0.01% crystal violet in 3% acetic acid), and leukocytes were counted under a light microscope.

Expression of cell adhesion molecules (CAMs)

Expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin was determined by whole-cell ELISA. Briefly, HUVEC monolayers were treated with each compound at the indicated concentrations for 6 h, followed by treatment with high glucose (25 mM) for 24 h, and fixed in 1% paraformaldehyde. After washing three times, mouse anti-human monoclonal antibodies (VCAM-1, ICAM-1, E-selectin, Temecula, CA, USA, 1:50 each) were added, and cells were incubated for 1 h (37 °C, 5% CO₂). Cells were then washed, treated with peroxidase-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO) for 1 h, washed three times, and then developed using 3,3′-diaminobenzidine substrate (Sigma, St. Louis, MO). All measurements were performed in triplicate wells.
Cell-Cell adhesion assay

Adherence of monocytes to endothelial cells was evaluated by fluorescent labeling of monocytes. Briefly, monocytes were labeled with 5 µM Vybrant DiD for 20 min at 37°C in phenol red-free RPMI containing 5% fetal bovine serum. Following two washings, cells (1.5 × 10^6/ml, 200 µl/well) were resuspended in adhesion medium (RPMI containing 2% fetal bovine serum and 20 mM HEPES) and added to confluent monolayers of HUVECs in 96-well plates, which were treated for 6 h with each compound followed by high glucose (25 mM for 24 h). Fluorescence of labeled cells was measured (total signal) using a fluorescence microplate reader (Tecan Austria GmbH, Austria). After incubation for 1 hour at 37°C, non-adherent cells were removed by washing four times with pre-warmed RPMI and the fluorescent signals of adherent cells were measured using previously described methods. The percentage of adherent monocytes was calculated using the formula: % adherence = (adherent signal/total signal) x 100 as described (6-7).

RNA preparation and real time qRT-PCR

HUVECs were grown in six-well plates and incubated with each compound for 6 h, followed by HMGB1 25 mM for 24 h. The High pure RNA isolation kit, from Roche Diagnostics was used for isolation of RNA from cell cultures, and RNA quality was tested by measuring the ratio 260/280 nm in a UV–spectrophotometer. For each sample, 0.5 µg of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics).

Real-time PCR analysis was performed using the LightCycler® 96 System (Roche Diagnostics,
Mannheim, Germany) using FastStart Essential DNA Green Master (Roche Diagnostics) according to the manufacturer’s instructions. GAPDH was used as an internal control. The relative quantification of mRNA expression was calculated as a ratio of the target gene to GAPDH. Specific sense and anti-sense primers used were as follows, respectively: MCP-1, sense: 5′-TGCAGAGGCTCGAGCTA-3′; anti-sense: 5′-CAGGTGGTCCATGGAATCTGA-3′; IL-8, sense: 5′-ACTGAGAGTGATTGAGATGGAC-3′; antisense: 5′-AACCTCTGCACCAGTTTC-3′, GAPDH, sense: 5′-GTCTTCACTACCATGGAGAAGG-3′; antisense: 5′-TCATGGATGACCCCTTGCCAG-3′.

\textit{H}_2\textit{O}_2 \textit{release assay}

Extracellular production of \textit{H}_2\textit{O}_2 was quantified using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes; Eugene, OR, USA) according to the manufacturer's recommendations. The cells were washed twice with ice-cold PBS and harvested by microcentrifugation and resuspended in a Krebs-Ringer phosphate (KRPG) solution; 100 µl of the reaction mixture (50 µM Amplex Red reagent containing 0.1 U/ml HRP in KRPG) was added to each microplate well and then prewarmed at 37 °C for 10 min. Then, the reaction was started by addition of resuspended cells in 20 µl of KRPG. Fluorescence readings became stable within 30 min of starting the reaction equipped for absorbance at ∼560 nm (Multiskan, Thermo Labsystems Inc, Franklin, MA, USA). A reagent \textit{H}_2\textit{O}_2 standard curve was used for calculation of \textit{H}_2\textit{O}_2 concentration.

\textit{Preparation of cytoplasmic and nuclear extracts}

The cells were harvested rapidly by sedimentation and nuclear and cytoplasmic extracts were prepared.
on ice, as previously described (8). Cells were harvested and washed with 1 ml buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 19 mM KCl) for 5 min at 600 g. The cells were resuspended in buffer A and then centrifuged at 600 g for 3 min, resuspended in 30 µl buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42M NaCl, 1.5mM MgCl₂, 0.2 mM EDTA), rotated for 30 min at 4°C, and then centrifuged at 13,000 g for 20 min. The supernatant was used as a nucleus extract. The nucleus and cytosolic extracts were then analyzed for protein content using Bradford assay.

Western blotting

Total cell extracts were prepared by lysing the cells and protein concentration was determined using Bradford assay methods. Equal amounts of protein were separated by SDS-PAGE (10%) and electroblotted overnight onto an Immobilon membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with 5% low-fat milk-powder TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and then incubated with NF-κB p65 for 1.5 h at room temperature (1:1000, Santa Cruz, CA, USA), followed by incubation with horseradish-peroxidase-conjugated secondary antibody and ECL-detection according to the manufacturer's instructions. β-actin (1:1000, Santa Cruz) or lamin A/C (1:1000, Santa Cruz) was used as a loading control for cytoplasmic or nuclear extracts, respectively.

Immunofluorescence staining

HUVECs were grown to confluence on glass cover slips coated with 0.05% Poly-L-Lysine in complete media containing 10% FBS and maintained for 48 h. Cells were then stimulated with high glucose (25 mM) for 1
h with or without prior treatment with each compound for 2 h. After several washes with PBS, cells were fixed in 4% formaldehyde in PBS (v/v) for 15 min at room temperature, and for immunostaining, cells were permeabilized in 0.05% Triton X-100 in PBS for 15 min, and blocked in blocking buffer (5% BSA in PBS) overnight at 4°C. Cells were incubated with primary rabbit monoclonal NF-κB p65 antibody, anti-rabbit alexa 488 overnight at 4°C. Nuclei were counterstained with 4,6-diamidino-2-phenylindole dihydroChloride (DAPI). Cells were then visualized by confocal microscopy at a 63× magnification (TCS-Sp5, Leica Microsystems, Germany).

Statistical Analysis

Results are expressed as mean ± standard error of mean (SEM) of at least three independent experiments. Statistical significance was determined using analysis of variance (ANOVA; SPSS, version 14.0, SPSS Science, Chicago, II, USA) and p-values less than 0.05 (p < 0.05) were considered significant.

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Supplementary References

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