Phenolic compounds from *Morus nigra* regulate viability and apoptosis of pancreatic β-cells possibly via SERCA activity

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Supporting Information:

Details of Experimental Procedures

SERCA activity measurement

SR vesicles (1 mg prot./ml) were added to the assay mixture (40 mM HEPES pH 7.2, 0.1 M KCl, 5.1 mM $MgSO_4$, 2.1 mM ATP, 0.52 mM phosphoenolpyruvate, 1 mM EGTA, 0.15 mM NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase) and incubated for 2 minutes with phenolic compounds (10-70 μM) at 37 ºC, pH 7.2. The reaction was started by addition of 1 mM $CaCl_2$ to the reaction mixture. The absorbance was recorded at 340 nm with a microplate reader (Infinite M200, Tecan, Switzerland).

Labeling of SERCA1 in SR vesicles using FITC

After incubation with compounds, FITC-ATPase labeling ratio of approximately 0.5:1.0 was performed. Stock solution of FITC (6 mM) was prepared in dry dimethylformamide. SR (0.6 mg protein/ml) was diluted in the volume of 35 μl of a HEPES buffer (1M KCl, 0.25 M sucrose and 50 mM HEPES, pH 8.0). The final reaction mixture contained 2.5 ηM of FITC. The reaction mixture was left to stand at room temperature in dark for 1 h and consequently diluted with 250 μl of a Tris-buffer (0.2 M sucrose, 50 mM Tris–HCl, pH 7.0). Labeled samples were left to stand for 30 min at 37 °C and stored on ice until detection. Labeled protein (15 μg) was added

to 1ml of Tris-buffer (5 mM MgSO4,100 mM KCl, 25 µM EGTA and 50 mM Tris–maleate buffer, pH 7.0, 25°C).

Culturing of beta-cells

INS-1E insulinoma pancreatic beta-cell line (kindly provided by Prof. Claes Wollheim, University of Geneva) was cultured in RPMI 1640 (11 mM glucose, Sigma Aldrich). RPMI 1640 was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM Na-pyruvate, 55 µM 2-mercaptoethanol, 10 mM HEPES, 1% non-essential amino acids, and 10% fetal bovine serum, pH 7.0-7.4. The cells were grown in a humidified incubator containing 5% CO2 at 37 °C. Lysis buffer contained 0.2 ml per $1x10^6$ cells/25 cm^2 flask containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/ml leupeptin. Cells were incubated with the IC_{50} concentrations of individual compounds for 24 h and then submitted to lysis in ice-cold lysis buffer. The cells were homogenized by passing the lysate through a syringe needle. The lysates were kept on ice for 10 min and then centrifuged for 10 min at 20 160 RCF and kept at -30 °C until further use.

Separation of proteins

Laemmli buffer (25mM Tris, 1% SDS, 0.192M glycine, 1% bromphenol blue, pH 6.8) containing freshly added 5% mercaptoethanol was added to the protein samples. The samples were loaded onto an SDS–polyacrylamide gel (10% separating, 4% stacking gel). The separation was performed for 0.5 h at 50 V and then 1.5 h at 150 V. After SDS-PAGE, the proteins were transferred to immobilon-P polyvinylidene fluoride (PVDF) membrane by semidry immunoblotting (250 mA for 30 min). All incubations were performed in PBS, 0.1% Tween. The membrane with transferred proteins was blocked with 5% fat-free dry milk for 2 h and then exposed to the monoclonal mouse IgG antibody against SERCA2 (IID8, AB 630230, sc-53010, Santa Cruz, USA 1:1 000 in 2.5%, fat-free dry milk). All membranes were incubated with mouse anti-β-actin monoclonal antibody (AB 2714189, sc-47778, Santa Cruz, USA, 1:10 000). Goat anti-mouse IgG-HRP (AB631736, sc-2005, Santa Cruz, USA 1:10 000 in 2.5%, fat-free dry milk) was used as a secondary antibody for both SERCA2b and β-actin. The bands were visualized using luminol (sc-2048, Santa Cruz, USA) as a chemiluminescent probe and analyzed by Amersham Imager 600 (GE Health Care Europe GmbH, Freiburg, Germany).

Cytotoxicity assay (MTT)

INS-1E cells were seeded in 96-microwell plates at a density of $5x10^4$ cells per well. The cells were pre-incubated for 24 h with or without different concentrations of individual phenolic compounds (1-200 µM), 5% CO_2, at 37 °C. MTT was added to achieve the final concentration of 0.5 mg/ml and after incubation over a period of 4 h, solubilization buffer (10% SDS in 0.01 M HCl) was added and let to stand for 15-17 h to solubilize the formazan formed.

Detection of apoptosis

INS-1E cells were harvested and resuspended in cold Krebs-Ringer Bicarbonate Buffer (KRBH) comprised of 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, and 0.1% (w/v) bovine serum albumin. The cells were counted and the cell suspension was adjusted to 1×10^6 cells/ml with KRBH buffer. FITC Annexin (5 µl) and propidium iodide (2 µl of the 100 µg/ml PI stock solution) were added to each 100 µl sample cell suspension and the cells were incubated for 15 min in the dark. Finally, 400 µl of KRBH were added and the stained cells were analyzed by flow cytometry (Beckman Coulter, USA) using FL1 and FL3 channels. A total of 5000-gated events were analyzed per sample by flow cytometry.

Release of insulin

The secretory responses to glucose were measured in INS-1E cells, after 24 h-preincubation of beta-cells with individual compounds the cells were washed with glucose free KRBH buffer. Next, they were incubated for 30 min at 37 °C in glucose free KRBH buffer and then for 30 min at 37 °C in KRBH buffer supplemented with 16.7 mM glucose. After incubations, the supernatants were collected for insulin secretion, detected by RIA kit (Mercodia, Sweden) using rat insulin as standard.