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Molecular mechanism of ^{18}F-FDG uptake reduction induced by genipin in T47D cancer cell and role of uncoupling protein-2 in cancer cell glucose metabolism☆

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ABSTRACT

Introduction: Compounds that modulate cancer cell glucose metabolism could open new opportunities for antitumor therapy and for monitoring response using ^{18}F-FDG PET. Genipin, a natural dietary compound that blocks uncoupling protein 2 (UCP2)-mediated mitochondrial proton leakage, is a potential anticancer agent. We investigated the effect of genipin on glucose metabolism and the mitochondrial function of cancer cells.
Methods: Breast and colon cancer cells were assessed for effects of genipin on ^{18}F-FDG uptake. T47D breast cancer cells were further evaluated for time-dependent and dose-dependent effects on ^{18}F-FDG uptake, lactate release, oxygen consumption rate (OCR), reactive oxygen species (ROS) production, and mitochondrial membrane potential. The effects of UCP2 knockdown were evaluated using specific siRNA.
Results: Cancer cells displayed significant reductions in ^{18}F-FDG uptake by genipin. T47D cells showed the greatest reduction to 32.6 ± 1.0% of controls by 250 μM genipin. The effect occurred rapidly, reaching a plateau by 1 h that lasted up to 24 h. The effect was dose-dependent with a half-inhibitory concentration of 60.8 μM. An accompanying decrease in lactate release was consistent with reduced glycolytic flux. OCR was significantly decreased by genipin to 82.2 ± 11.4% of controls, and ROS generation was increased to 156.7 ± 16.0%. These effects were largely reproduced by UCP2 knockdown with specific siRNA.
Conclusions: Genipin decreased cancer cell ^{18}F-FDG uptake by reducing both glycolytic flux and mitochondrial oxidative respiration. This effect appeared to occur by blocking the ability of UCP2 to dissipate energy and restrict ROS production through proton leakage.

1. Introduction

Malignant cells have a heightened rate of glucose utilization that is associated with a shift in metabolism from mitochondrial respiration to glycolytic flux. This cancer hallmark is exploited by positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) for tumor detection and evaluation of treatment response [1], and is a promising target for cancer treatment [2]. Yet few cancer metabolism-targeting drugs to date have entered the clinical arena. Nature provides a largely unexplored source of bioactive molecules with anticancer potential. Natural compounds offer favorable bioavailability profiles and tolerability compared to synthetic drugs [3,4].

Genipin is a natural agent extracted from gardenia fruits that is traditionally used in herbal medicine for treating inflammation and jaundice [5,6]. Searching for lead compounds that can improve β-cell function, Zhang and coworkers found that gardenia extract stimulates insulin secretion by pancreatic islets derived from wild-type mice but not mice deficient in the mitochondrial membrane component UCP2 [7]. This observation led to the discovery that genipin is an inhibitor of UCP2 function [7]. The growth and survival of cells, including cancer cells, depends on mitochondrial function [8], which generates ATP through a complex process of controlled oxidative respiration [9]. This process pumps protons into the intermembrane space and UCP2 allows free re-entry of the protons back into the mitochondrial matrix (proton leak), dissipating the intermembrane proton gradient.

Although UCP2 was initially investigated for its role in obesity and diabetes [7,10–12], various cancer types have been found to have elevated UCP2 expression [13–15]. UCP2 expression in colon cancer correlates with degree of malignancy and metastasis [15,16]. These findings suggest that expression of this protein is a functional or survival

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advantage under certain conditions. In immune cells, UCP2 has a major role in limiting reactive oxygen species (ROS) production [17]. UCP2 may therefore confer protection on cancer cells from the adverse effects of elevated mitochondrial ROS production [18]. This possibility is supported by the finding that UCP2 promotes chemoresistance of cancer cells [19].

Given the ability of UCP2 to dissipate energy and restrict ROS production via mitochondrial membrane proton leakage, this protein may be involved in the Warburg effect of cancer cells. Investigating how natural agents influence tumor ^{18}F-FDG uptake and exploring their mechanisms may suggest new cancer treatments [20] and opportunities for monitoring treatment response with PET imaging [21]. In this study, we tested the hypothesis that genipin would reverse the Warburg effect of cancer cells by suppressing UCP2 function, and response would reduce ^{18}F-FDG uptake.

2. Materials and methods

2.1. Cell culture and reagents

T47D, MDA-MB-435, MDA-MB-231 and MCF7 human breast cancer cells and CT26, HCT116 and HT29 colon cancer cells were from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Cells were split 2–3 times a week and experiments were performed when confluence reached 80%. All reagents including genipin were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

2.2. Cellular ^{18}F-FDG uptake measurement

Cells in 12-well plates were incubated at 5% CO_2 and 37 °C for 40 min with 370 kBq of ^{18}F-FDG added to culture medium. Cells were washed, lysed, and measured for radioactivity as previously described [22].

2.3. Cellular oxygen consumption rate

Oxygen consumption rate (OCR) was measured as previously described [22]. Briefly, Seahorse XF24 24-well plates seeded with 60,000 cells per well were equilibrated for 1 h with serum-free RPMI 1640 (no sodium bicarbonate; pH 7.4), and oxygen concentration was measured on a Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience North Billerica, MA, USA). After basal OCR was determined, the following agents were sequentially added to the media: oligomycin (1.2 μM) to inhibit complex V, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 4 μM) to uncouple proton gradient, and antimycin A (10 μM) to inhibit complex III.

2.4. Lactate production assays

Lactate concentration (mU/mg) in culture media was measured using Cobas assay kits (Roche/Hitachi, Mannheim, Germany) with a calibrated standard curve as previously described [22].

2.5. Measurement of intracellular ROS

Intracellular ROS concentration was quantified using CM-H2DCFDA (Molecular Probes, Invitrogen; Carlsbad, CA, USA) as previously described [22]. Cells incubated with dye for 30 min were washed and fluorescence recorded on a BioTek FLx800 96-welled microplate fluorescence reader (Fisher Scientific; Waltham, MA, USA) with excitation/emission wavelengths of 485 nm/530 nm.

2.6. Western blots for UCP2 expression

Cells seeded on 100-mm plates were lysed with cold lysis buffer with PRO-PREPTM (Intron Biotechnology; Korea) and protease inhibitor. After centrifugation at 14,000*g* for 10 min at 4 °C, supernatants were transferred to new tubes. Protein concentration was determined according to the Bradford method. Samples were boiled for 5 min, separated by electrophoresis on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline and polysorbate-20 for 1 h at room temperature, membranes were incubated overnight at 4 °C with antibody against UCP2 (Abcam; Cambridge, MA, USA; 1:500 dilution) or β-actin (Santa Cruz Biotechnology; Dallas, TX, USA; 1:1000 dilution), followed by antimouse IgG horseradish peroxidase-linked secondary antibody (Cell Signaling Technology; Danvers, MA, USA; 1:2000 dilution) at room temperature for 1 h. Immunoreactive protein was detected by chemiluminescence, and band intensities were quantified using Bio-Rad Laboratories Quantity One software (Philadelphia, PA, USA).

2.7. UCP2 knockdown with specific siRNA

UCP2-specific siRNA in a pool of 3 target-specific siRNAs of 20–25 nucleotides designed to knockdown human UCP2 and nontargeting scrambled siRNA were obtained from Santa Cruz Biotechnology. Cells at 60–80% confluency were transfected with siRNA (20 nM) using Lipofectamine RNAiMax reagent (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 48 h prior to analysis.

2.8. Statistical analysis

All data are presented as mean ± SD of samples. Significance in differences between groups was analyzed by Student's *t*-tests for two groups and ANOVA with Tukey post hoc tests for three or more groups. p Values <0.05 were considered statistically significant.

3. Results

3.1. Expression of UCP2 protein in breast and colon cancer cells

Western blots showed expression of UCP2 protein in T47D, MDA-MB-435, MDA-MB-231 and MCF7 breast cancer cells and HCT116 and HT29 colon cancer cells. UCP2 protein bands were observed from T47D and HCT116 cells with intensities varying by cell type (Fig. 1A).

3.2. Genipin suppresses ^{18}F-FDG uptake by breast and colon cancer cells

Treatment for 24 h with genipin resulted in significant reductions in ^{18}F-FDG uptake in all breast and colon cancer cells tested except MCF7 cells. T47D cells showed the greatest reduction in ^{18}F-FDG uptake to 49.1 ± 1.5% of controls by 100 μM genipin. Treatment with 100 μM genipin for 24 h reduced ^{18}F-FDG uptake of MDA-MB-435 cells to 88.3 ± 3.4%, CT26 cells to 81.2 ± 2.8%, HCT116 cells to 72.8 ± 9.5% and HT29 cells to 66.7 ± 7.8% of controls. ^{18}F-FDG uptake by MDA-MB-231 and MCF7 cells was not significantly influenced by genipin (Fig. 1B).

3.3. Dose-dependent and time-dependent suppression of ^{18}F-FDG uptake and lactate production

Dose dependence experiments at 24 h exposure of T47D cells to different concentrations of genipin demonstrated reduced ^{18}F-FDG uptake to 70.9 ± 4.0% of controls at 50 μM genipin. Uptake further decreased to 52.6 ± 3.9% of controls at 100 μM genipin with a plateau reached at 200 μM of genipin (Fig. 2A). The sigmoidal dose–response curve showed a half-inhibitory concentration (IC_{50}) of 60.8 μM (95% confidence interval, 26.1 to 141.0 μM). Time courses of genipin effects revealed an acute