Effects of curcumin on cancer cell mitochondrial function and potential monitoring with $^{18}$F-FDG uptake

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Abstract. A better understanding of how curcumin influences cancer cell biology could help devise new strategies to enhance its antitumor effect. Many curcumin actions are proposed to occur by targeting mitochondrial function, among which glucose metabolism and reactive oxygen species (ROS) production are pivotal. However, little is known of how curcumin influences cancer cell glucose metabolism. We thus evaluated the effect of curcumin on cancer cell glucose metabolism and mitochondrial function, and further investigated whether these responses could be modified to enhance the anticancer potency of the compound. MCF-7 breast cancer cells treated with curcumin were measured for $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) uptake, lactate production, hexokinase activity, oxygen consumption rate (OCR), ROS production and mitochondrial membrane potential (MMP). Activation of signaling pathways was evaluated by western blots, and cell survival was assessed with sulforhodamine B assays. Curcumin stimulated a 3.6-fold increase of $^{18}$F-FDG uptake in MCF-7 cells, along with augmented hexokinase activity and lactate efflux. This was accompanied by significantly suppressed cellular OCR, consistent with a metabolic shift to glycolytic flux. Inhibiting this metabolic response with 2-deoxyglucose (2-DG) blocked curcumin-induced mTOR activation and resulted in a greater anti-proliferative effect. Curcumin-induced MMP depolarization led to reduced ROS production, which may hinder the anticancer effect of the compound. Intracellular ROS was completely restored by adding Cu$^{2+}$, which can bind and modify the curcumin's physico-chemical property, and this resulted in a marked potentiation of its anti-proliferative effect. Thus, curcumin suppresses cancer cell MMP and ROS generation, and this response is accompanied by stimulated $^{18}$F-FDG uptake via shifting of metabolism from mitochondrial respiration to glycolytic flux. These mitochondrial and metabolic responses may provide potential targets that can help enhance the anticancer action of curcumin.

Introduction

Curcumin, a biocompatible polyphenol of the dietary spice turmeric, has been shown to exhibit anticancer effects in multiple cancers including those of the breast, ovary, colon and brain (1). However, the clinical application of curcumin has been hindered to date, not only due to its low solubility and bioavailability, but also by limitations in its anticancer potency. It is, therefore, important to better understand how curcumin influences the biology of cancer cells so that new strategies can be devised to enhance its antitumor effect.

Recent evidence points to mitochondria as the target for many of the beneficial effects of curcumin (2). A major function of mitochondria is to generate energy by utilizing substrates. Although malignant cells undergo reprogramming of energy metabolism toward aerobic glycolysis (3), they also depend on mitochondrial oxidative metabolism for proliferation and survival. Thus, cancer cell glucose metabolism is gaining interest as a promising target for cancer treatment (4), and is exploited by positron emission tomography (PET) with $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) for monitoring treatment efficacy (5). Another important mitochondrial function is the production of reactive oxygen species (ROS), which mediates crucial cellular tasks including signal transduction and induction of apoptosis (6). Hence, cancer-specific modifications to ROS and anti-oxidants are also considered a potential target for therapy (6).

A link between curcumin action and glucose metabolism is suggested by observations in skeletal muscle (7) and myotubes (8) that curcumin can stimulate glucose uptake. However, how cancer cell glucose metabolism is influenced by curcumin, and whether suppressing this response affects its anticancer action has not been investigated. Furthermore, although curcumin is through to induce apoptosis of cancer cells through oxidative stress (9,10), it is not known whether suppressing the ROS scavenging activity of curcumin (11) can enhance its antitumor effect. This could be relevant for...
the development of newer curcumin analogs with strengthened pro-oxidant properties for improved therapeutic response (12,13). In addition, since cancer cells heavily rely on glucose utilization, understanding how metabolism-targeting drugs modulate tumor 18F-FDG uptake may not only help uncover new strategies for cancer treatment but could also expand the role of PET imaging for monitoring treatment response.

In the present study, we examined how curcumin influences cancer cell glucose metabolism and mitochondrial function, including 18F-FDG uptake, lactate production, hexokinase activity, oxygen consumption, mitochondrial membrane potential (MMP) and ROS production. We further investigated whether restricting glycolysis with 2-deoxyglucose (2-DG) and addition of Cu2+, known to reduce curcumin's anti-oxidant property (14-16), can enhance the anticancer action of the compound.

Materials and methods

Cancer cell lines and culture. MCF-7 human breast cancer and CT26 colon cancer cell lines were from the American Type Culture Collection (ATCC; Rockville, MD, USA), and maintained in MEM (MCF-7 cells) or RPMI-1640 media (CT26 cells) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator at 37˚C, 5% CO2. Cells grown in a monolayer were routinely split 2 times a week, and experiments were performed at 48 h after seeding. For cell treatment, stock solutions of curcumin dissolved in DMSO, 2-DG dissolved in phosphate-buffered solution (PBS), and CuSO4 dissolved in 0.05 M Tris-HCl (pH 7.4) were used.

Reagents and antibodies. Curcumin, 2-DG, copper sulfate (II) (CuSO4), oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazide (FCCP) and antimycin-A were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). CM-H2DCFDA (5-(and-6)-chloromethyl-2′9,7′-dichlorodihydrofluorescein diacetate, acetyl ester) and MitoTracker Red FM was from Invitrogen Life Technologies (Grand Island, NY, USA). Antibodies against phosphorylated mammalian target of rapamycin (p-mTOR) and phosphorylated extracellular-signal-regulated kinase (p-ERK), and anti-rabbit and anti-mouse secondary antibodies were from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibody against β-actin was from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit polyclonal antibody against glucose transporter 1 (GLUT1) was from Abcam (Cambridge, MA, USA).

Sulforhodamine B (SRB) assay. Surviving cell content following treatment was evaluated by SRB assays. Briefly, cells seeded overnight on a 96-well plate were treated for indicated durations of time, and cell monolayers were fixed with 10% (wt/vol) trichloroacetic acid at 4˚C. After cells were stained with SRB dye for 30 min, excess dye was removed by washing repeatedly with 1% (v/v) acetic acid. Protein-bound dye was finally dissolved in 10 mM Tris base solution and subject to spectrophotometric measurement of absorbance at 510 nm using a microplate reader.

FDG uptake measurement. Cells in 12-well plates were incubated with 370 kBq (10 µCi) of the radiolabeled glucose analogue 18F-FDG for 40 min at 5% CO2, 37˚C. After rapid washing twice with cold PBS, cells were lysed with 500 µl of 0.01 N NaOH and cell-associated radioactivity was measured on a high-energy γ-counter (Perkin-Elmer, Waltham, MA, USA). Radio-uptake levels were corrected for cell content as assessed by Bradford protein assays.

Lactate production. L-lactate production was measured from 100 µl of culture medium using a Cobas assay kit (Roche/Hitachi) following the manufacturer's instructions. In the assay, lactate is enzymatically converted to pyruvate and hydrogen peroxide. Hydrogen peroxide then undergoes an enzymatic reaction to generate a colored dye that is measured by absorbance on a microplate spectrophotometer. Lactate concentration was calculated from a standard curve of serially diluted standards and expressed in mM/L.

Hexokinase activity. Cells homogenized in homogenizing buffer (50 mM triethanolamine and 5 mM MgCl2; pH 7.6) were centrifuged at 1,000 g for 5 min at 4˚C. The pellet mitochondrial fraction was isolated and 50 µl was mixed with 2.52 ml of reaction buffer containing 39 mM triethanolamine, 216 mM D-glucose, 0.74 mM adenosine 5′-triphosphate, 7.8 mM magnesium chloride, 1.1 mM β-nicotinamide adenine dinucleotide phosphate, and 2.5 units of glucose 6-phosphate dehydrogenase. The reaction mixture was repeatedly measured at 25˚C for spectrophotometric absorbance at 340 nm. Hexokinase activity was expressed as mM/L of protein, and one unit of activity was defined as the amount of hexokinase activity that phosphorylates 1 µmol of glucose per min at 25˚C.

Oxygen consumption rate (OCR). Cells were seeded in 100 µl of MEM media at a density of 5x104 cells/well on analyzer plates. A Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience Inc., North Billerica, MA, USA) with a solid state sensor probe analyzed the concentration of oxygen dissolved in the media at 2-sec intervals. Following measurement of basal OCR level, 1.2 µM oligomycin (ATP synthase inhibitor), 4 µM FCCP (proton gradient uncoupler), and 10 µM antimycin A (mitochondria electron transport inhibitor) were sequentially added to assess their effects on OCR. Cell density and working concentrations for analysis was optimized according to the manufacturer's manual. OCR was automatically calculated, recorded, and plotted by Seahorse XF24 software version 1.8.

MMP measurement. Cells were seeded at densities of 5x104 per well in a 96-well black plate with a transparent bottom. Culture media was removed and wells were replenished with 100 µl of MEM media containing 500 nM of MitoTracker Red FM (Invitrogen), a fluorescent dye that stains mitochondria of live cells as a function of mATP. Cells were incubated for 30 min at 37°C in 5% CO2, and then washed with 100 µl of warmed PBS per well. Fluorescence remaining in each well was measured on a microplate reader using 594 nm excitation and 642 nm emission wavelengths.

Intracellular ROS production. To quantify intracellular ROS, cells were seeded at densities of 5x10^4/well in a 96-well black plate. Culture media with treating agents was removed and wells were replenished with 100 µl of MEM media containing 10 µM

of CM-H$_2$DCFDA (Molecular Probes-Invitrogen), a cell-permeant fluorescent indicator for intracellular ROS. The probe is non-fluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell, which can be detected by monitoring the increase in fluorescence. Cells were incubated with the probe for 30 min at 37°C in 5% CO$_2$, after which excessive dye was removed and cells were washed with 100 µl of warmed PBS per well. Fluorescence remaining in each well was measured on a GloMax® microplate reader (Promega, Seoul, Korea) with 490 nm excitation and 510-570 nm emission wavelengths.

**Western blot analysis.** After cells were lysed with cold PRO-PREP lysis buffer (iNtRON Biotechnology, Seoul, Korea), 30 µg of total cell lysate was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk in Tris-buffered saline and oxidation occurs within the cell, which can be detected by monitoring the increase in fluorescence. Cells were incubated with the probe for 30 min at 37°C in 5% CO$_2$, after which excessive dye was removed and cells were washed with 100 µl of warmed PBS per well. Fluorescence remaining in each well was measured on a GloMax® microplate reader (Promega, Seoul, Korea) with 490 nm excitation and 510-570 nm emission wavelengths.

**Statistical analysis.** All experiments were repeated two or three separate times, and the mean ± SD of triplicate samples from a single representative experiment is presented unless otherwise specified. Student's t-tests were used to evaluate the statistical significance of measurements between groups, and P-values <0.05 were considered statistically significant.

**Results**

*Curcumin dose-dependently stimulates cancer cell $^{18}$F-FDG uptake.* Curcumin dose-dependently stimulated $^{18}$F-FDG uptake of MCF-7 and CT26 cancer cells. Hence, $^{18}$F-FDG uptake corrected for cell content was substantially increased to 250±13 and 193±5% of control levels by exposure to 30 µM curcumin for MCF-7 cells and 10 µM curcumin for CT26 cells for 24 h (Fig 1A). Time course experiments in MCF-7 cells disclosed that stimulation of $^{18}$F-FDG uptake by 30 µM curcumin began as soon as 2 h, with further increases that reached 362.5±12.5% of control level by 24 h (Fig. 1B). This metabolic effect occurred at a curcumin dose that only mildly reduced surviving cell content (85.1±2.7% of controls; Fig. 1C).

*Curcumin stimulates glycolytic flux and suppresses mitochondrial respiration.* In MCF-7 cells, treatment with 30 µM curcumin for 24 h caused a significant elevation of lactate release to 144.6±3.9% of controls (Fig. 2A), consistent with a shift of metabolism toward glycolytic flux. To evaluate the contribution of the major mediators of cellular $^{18}$F-FDG uptake, we measured hexokinase activity and membrane GLUT1 expression. As a result, 30 µM curcumin for 24 h was found to significantly augment mitochondrial hexokinase activity to 146.0±34.5% of controls (Fig. 2B). In contrast, immunoblotting showed that curcumin had no significant influence on the level of membrane GLUT1 expression (data not shown).

We next evaluated whether the shift toward glycolytic flux by curcumin was associated with suppression of mitochondrial oxidative respiration. Extracellular flux analysis of MCF-7 cells demonstrated that treatment with 30 µM curcumin for 6 h
caused a significant reduction of basal OCR to 67.5±1.4% of untreated controls (Fig. 2C). Inhibition of ATP synthase with oligomycin reduced OCR of control and curcumin-treated cells to similar levels, consistent with comparable amounts of proton leakage. Uncoupling of the proton gradient with FCCP caused a substantial augmentation of OCR for control cells but not for curcumin-treated cells. This indicates that maximal mitochondrial respiration is markedly suppressed by curcumin (Fig. 2C). Mean OCR over 6 h following treatment with curcumin was decreased to 67.6±0.4% of that following addition of vehicle (Fig. 2C).

**Glycolysis inhibition with 2-DG potentiates the anticancer effect of curcumin.** Given the shift toward glycolytic metabolism induced by curcumin, we next investigated whether additional blocking of glycolysis would potentiate its anticancer effect. 2-DG was first confirmed, as expected, to markedly diminished MCF-7 cell 18F-FDG uptake. When used in combination, 2-DG also completely blocked the ability of curcumin to stimulate 18F-FDG uptake. Hence, in the presence of 2-DG, 18F-FDG uptake was substantially reduced to 20.9±1.3% of untreated controls cells even when curcumin was added (Fig. 3A).

When antitumor effects were examined, curcumin and 2-DG added separately caused only mild reductions of MCF-7 cell content to 87.1±2.9 and 80.0±2.0% of controls, respectively. In comparison, co-treatment with curcumin and 2-DG reduced cell survival more efficiently to 67.1±1.9% of controls (Fig. 3B). We further evaluated how MAP kinase and mTOR signaling are affected by treatment. Immunoblotting revealed that curcumin, either alone or in combination with 2-DG, completely abrogated ERK activation (Fig. 3C). Activation of mTOR was not influenced by curcumin or 2-DG alone, but was significantly decreased to 52.5±11.1% of untreated control level when the two agents were combined (Fig. 3C).

**Curcumin depolarizes cancer cell MMP and reduces intracellular ROS generation.** To investigate how curcumin affects mitochondrial function, we measured MMP, which is critically involved in maintaining the mitochondrial respiratory chain and in avoidance of cell death. The results showed that exposure to curcumin for 24 h decreased MMP level of MCF-7 cells, in a manner inversely correlating to the applied dose. With a concentration of 30 µM, MMP dropped to 31.2±3.1% of control level (Fig. 4A). The reduction of MMP began as
soon as 2 h of treatment and continued to decrease further over 24 h (Fig. 4A).

Intracellular ROS of MCF-7 cells were also dose-dependently reduced by curcumin, and the pattern was similar to the
decrease of MMP. Treatment with 30 µM curcumin for 24 h reduced ROS level to 52.2±14.5% of controls (Fig. 4B). Time course experiments disclosed a rapid decline of ROS level to 48.6±6.2% of controls by 2 h (Fig. 4B). ROS decreased slightly further to 37.1±3.2% of controls by 6 h, but slightly recovered thereafter to 46.5±3.4% of controls by 24 h (Fig. 4B). Blocking the anti-oxidant property of curcumin markedly potentiates its anticancer effect. Finally, given the ROS-lowering property of curcumin in the literature as well as in our experimental data, we evaluated whether suppressing the anti-oxidant property of curcumin could enhance its anti-cancer effect. This was achieved by adding 40 µM CuSO_4, which is reported to neutralize the anti-oxidant domains of curcumin. As a result, ROS level that was significantly reduced by 30 µM curcumin alone, was recovered to control levels in the presence of copper (Fig. 5A).

SRB assays showed that CuSO_4 alone caused no significant influence on MCF-7 cell survival in concentrations of up to 40 µM. However, when combined together, 40 µM CuSO_4 substantially potentiated the dose-dependent reduction of cell survival induced by curcumin. Hence, cell content that was reduced to 75.2±2.6% of controls by 40 µM curcumin alone was further reduced to 30.6±8.2% of controls in the presence of 40 µM CuSO_4, pointing to a synergistic anticancer effect. Non-linear analysis of the dose-response curve showed IC_{50} values of 118.0 and 37.5 µM for curcumin in the absence and presence of CuSO_4, respectively (Fig. 5B).

Discussion

The present study demonstrates that curcumin dose-dependently stimulates ^18^F-FDG uptake in MCF-7 breast cancer cells by shifting glucose metabolism toward glycolytic flux. This response is accompanied by a suppression of mitochondrial oxidative respiration and reduction of ROS production.

Curcumin is well-recognized for its anti-hyperglycemic effect through stimulation of glucose uptake in muscle tissue. However, little is known regarding its influence on glucose handling of malignant cells. Since curcumin is well-recognized to exert anti-proliferative and apoptotic effects on breast cancer cells (17), we were particularly interested in how glucose metabolism and mitochondrial function of these cells are affected. Our results revealed that curcumin substantially enhances glucose uptake of both MCF-7 and CT26 cells, but MCF-7 cells showed a more robust dose-dependent response. As these two cell types are divergent in genetic makeup and biological property, it is difficult to point to a single reason explaining the different dose-responses of glucose uptake by curcumin. Nonetheless, this finding and our interest in breast cancer led us to select MCF-7 cells for further experiments. Although this study did not include normal cells, a previous study showed that curcumin and derivatives had minimal cytotoxic effects on normal mammary epithelial MCF-10A cells (18). In MCF-7 cells, the metabolic response began within a couple of hours of exposure, and was linked to stimulation of glycolytic flux, as evidenced by augmentations of lactate production and mitochondrial hexokinase activity. The significant reduction of OCR accompanying this response indicates a suppression of mitochondrial oxidative respiration. Curcumin further caused a marked blunting of the normal OCR response to FCCP, consistent with a severe reduction of maximal respiratory capacity. Tumor cells need to balance their metabolism in a manner that maintains a supply of energy production sufficient for continued growth and survival. Measurements of intracellular ATP would have strengthened our case but this was not performed in the present study. Taken together, our stimulation of glycolytic flux by curcumin appears to be a compensatory response of cancer cells attempting to maintain energy homeostasis in the face of mitochondrial dysfunction.

The dependence of malignant cells on a shift of energy metabolism to glycolytic flux to adapt to curcumin-induced suppression of mitochondrial respiration could provide a potential target for enhanced therapeutic efficacy. We therefore tested the effect of 2-DG, a glucose analogue that is taken up by transporters and competitively inhibits glycolysis at the phosphoglucosomerase level (19). The results showed that stimulation of glycolysis by curcumin was completely blocked by 2-DG. Importantly, this had a clear additive effect on reducing cell survival compared to that achieved by curcumin alone. This finding is reminiscent of a recent observation that mitochondria targeted cationic agents reduced cancer cell
Curcumin can exhibit bifunctional anti-oxidant properties related to its ability to directly react with ROS and to indirectly induce expression of anti-oxidant proteins. Part of the curcumin molecule acts as chelator of positively charged metals, which play an important role in free radical trapping activity (2). A factor thought to play a major role in the exhibition of either anti-oxidant or pro-oxidant properties by curcumin is binding of metal ions. Based on our finding that ROS was lowered by curcumin, we examined whether addition of Cu²⁺ could enhance the pro-oxidant property, and thereby the anticancer effect, of the compound. The results revealed that when MCF-7 cells were simultaneously treated with CuSO₄, cell content was more effectively decreased than by curcumin alone. This finding is consistent with a previous report of potentiated curcumin-induced cancer cell cytotoxicity by transient metals such as Cu²⁺ (30,31). Curcumin is composed of two hydrophobic phenyl domains connected by a β-diketone moiety, which acts as a binding site for transient metals (14). Copper, an essential trace metal with many physiological and pathological activities, is one of the most redox-active metal ions in living cells. Curcumin and Cu²⁺ have strong interactions, and their binding modifies the physico-chemical properties of the drug. In fact, mobilization of endogenous copper has been proposed to stimulate pro-oxidant action and contribute to the cytotoxic effects of several polyphenolic compounds against cancer (16).

In our results, intracellular ROS was no longer reduced by curcumin when Cu²⁺ was present. Similarly, a recent study showed that curcumin-induced cancer cell apoptosis was increased by Cu²⁺ through ROS generation, whereas it was blocked by anti-oxidants (31). These findings are consistent with the notion that, despite its anti-oxidant properties, curcumin can exhibit pro-oxidant effects in the presence of elevated Cu²⁺ (32). Indeed, increased ROS production was implicated in the DNA damage caused by DNA-associated copper and phenolic compounds (33). ROS-mediated DNA degradation was also observed in cancer cells treated with curcumin in the presence of Cu²⁺ (34,35). These findings have led to the exploration of curcumin or synthetic curcuminoids complexed with metal for enhanced antitumor activity (36). While metal-curcumin complexes may act either as anti-oxidants or pro-oxidants, curcumin complexed with Cu²⁺ has been shown to exert pro-oxidant effects by generating ROS at a high free copper level in a reducing environment (37). Taken together, our results suggest that CuSO₄ blocks the direct ROS scavenging effect of curcumin.

In conclusion, curcumin treatment of MCF-7 cancer cells induces mitochondrial dysfunction that leads to a metabolic shift to glycolytic flux and reduced ROS generation. This leads to a significant increase in 18F-FDG uptake, suggesting the possibility that it could serve as an imaging biomarker of tumor response to curcumin. Furthermore, glycolysis inhibition with 2-DG and suppression of the compound's anti-oxidant property with Cu²⁺ may enhance the anticancer action of curcumin.

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