Treatment of Breast Tumor Cells In Vitro with the Mitochondrial Membrane Potential Dissipater Valinomycin Increases ^{18}F-FDG Incorporation

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Mitochondrial membrane potential is essential for adenosine triphosphate (ATP) synthesis by oxidative phosphorylation, and its abolition is an early event during apoptosis, a type of cell death commonly exhibited by tumor cells responding to treatment. Dissipation of mitochondrial membrane potential can be specifically induced using the K^+ ion channel–opening agent valinomycin and has been used in this study to determine how the loss of mitochondrial membrane potential could influence ^{18}F-FDG incorporation. **Methods:** MCF-7 cells were treated with valinomycin for 30 min, inducing loss of mitochondrial membrane potential as determined using flow cytometry with the JC-1 probe. ^{18}F-FDG incorporation, the initial rate of *O*-methyl-D-glucose incorporation (a measure of glucose transport), hexokinase activity and subcellular distribution, ATP content using bioluminescence, and lactate production were determined on control and valinomycin-treated cells. **Results:** A 30-min treatment of MCF-7 cells with 1 μmol of valinomycin per liter resulted in absence of red fluorescence from JC-1, indicative of dissipation of mitochondrial membrane potential. ^{18}F-FDG incorporation was significantly increased by 30 min of treatment with valinomycin and was still apparent after 3.5 h of incubation. Hexokinase activity and subcellular distribution were not significantly different between control cells and cells treated for 30 min with valinomycin. Glucose transport was moderately though significantly increased, and lactate production was also increased. **Conclusion:** Loss of mitochondrial membrane potential is associated with increased ^{18}F-FDG incorporation, glucose transport, and lactate production.

Key Words: ^{18}F-FDG; mitochondria; membrane potential; glucose transport; lactate

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PET using the tracer ^{18}F-FDG is becoming a routine tool in the early detection of tumor response to therapy. Tumors responding to therapy generally show a decreased incorporation of ^{18}F-FDG within the first 1 or 2 cycles of chemotherapy (*1*), a result believed to reflect a decreased number of viable tumor cells (*2*). However, several studies that have determined ^{18}F-FDG incorporation early after therapy, during cell response to damage, have shown that ^{18}F-FDG uptake can be increased in tumors that are subsequently shown to respond well to treatment (*3–5*) and in cell lines responding to ionizing radiation or chemotherapy (*6–11*).

It has been reported that an early increase in ^{18}F-FDG incorporation after treatment of cells using ionizing radiation corresponds to an increased level of apoptosis, a form of cell death frequently associated with response to cytotoxic anticancer treatment, including radiation therapy (*12*). Furuta et al. (*13*) determined ^{18}F-FDG uptake by xenografts 2–6 h after irradiation and found that the most radiation-sensitive tumor, an ependymoblastoma, which showed high levels of apoptosis, exhibited enhanced ^{18}F-FDG uptake after irradiation when compared with nonirradiated controls. Two other tumors that were far less sensitive to radiation and exhibited only low levels of apoptosis did not show enhanced ^{18}F-FDG uptake.

Apoptosis involves the loss of mitochondrial membrane potential followed by mitochondrial membrane swelling and release of cytochrome c (*14*). Loss of mitochondrial membrane potential is associated with changes in the energy status of cells (*15–18*) and so is likely to influence the incorporation of ^{18}F-FDG. The mitochondrial membrane can be depolarized by treating cells with the selective K^+ ion channel–opening agent valinomycin. To determine whether loss of mitochondrial membrane potential could contribute to clinical and experimental findings regarding early increases in ^{18}F-FDG incorporation after therapy, we measured ^{18}F-FDG incorporation by MCF-7 cells treated with valinomycin. We also determined how dissipation of the mitochondrial membrane potential modulated glucose transport, hexokinase activity, adenosine triphosphate (ATP) concentration, and lactate production.

MATERIALS AND METHODS

Materials

All chemicals were acquired from Sigma-Aldrich Chemical Co. unless otherwise stated.

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Cells and Treatment

MCF-7 cells were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 units of penicillin per milliliter, and 50 μg of streptomycin per milliliter (Invitrogen). The cells were grown in 75-cm^2 tissue culture flasks in a CO_2 incubator (5% CO_2:95% air) at 37°C and routinely passaged when confluent. Before each experiment, cells were seeded in 25-cm^2 tissue culture flasks. Cells in the requisite numbers of flasks were treated by the addition of new medium containing valinomycin in dimethyl sulfoxide (final concentration, 1 μmol/L) for 30 min. Control cells received fresh medium containing the same amount of dimethyl sulfoxide vehicle.

^{18}F-FDG Incorporation

Flasks of untreated and treated cells were incubated at 37°C with medium containing 1 kBq of ^{18}F-FDG per milliliter for 20 min. The medium was then removed, 100 μL were frozen for lactate determination, and the flasks were washed 5 times with 6 mL of ice-cold phosphate-buffered saline (PBS). Cells were then removed by the addition of 0.5 mL of trypsin and, after the addition of 0.5 mL of medium, were transferred to a microcentrifuge (Eppendorf) tube. After removal of 200 μL for ATP determination, radioactive counts were determined using a well counter. The cells were then pelleted by centrifugation at 400*g* for 5 min and were washed with 1 mL of PBS. The washed pellet was dissolved overnight in NaOH (1 mol/L). After neutralization with HCl (1 mol/L), protein content was determined using a bicinchoninic acid protein assay kit according to the manufacturer's instructions (Sigma-Aldrich). The results were expressed as counts per minute, per milligram of protein.

Glucose Transport

Glucose transport was determined by measuring the initial uptake of the nonmetabolized glucose analog ^{3}H-*O*-methyl glucose (OMG) (Amersham). Flasks of cells were incubated with fresh medium containing ^{3}H-OMG plus 0.1 mmol of OMG per liter for 10 s (uptake of OMG by MCF-7 cells is linear for at least 30 s (*19*)), after which 10 mL of ice-cold PBS were added and rapidly removed by inverting the flask. Three more rapid washes were performed, and then the cells were detached by incubation with trypsin and, after being neutralized with medium, were thoroughly mixed by working a 1-mL pipette up and down to break up clumps. Half the cell suspension was then added to 5 mL of Ultima Gold (PerkinElmer) scintillation fluid, and ^{3}H incorporation was determined on a scintillation counter. The other half was centrifuged at 400*g* for 5 min, the supernatant was discarded, and the pellet was resuspended in 1 mL of PBS. After further centrifugation, the pellet was resuspended in 0.1 mL of NaOH (1 mol/L). Protein content was then determined as described above.

Hexokinase Activity

Cell homogenate was prepared and cells fractionated (*20,21*). Flasks of treated and control cells were harvested by trypsinization and neutralization with medium. They were then placed in microcentrifuge tubes and centrifuged at 400*g* for 5 min at 4°C. After the supernatant had been discarded, the pellet was washed with PBS and then resuspended in 100 μL of homogenization buffer (Tris/HCl [10 mmol/L, pH 7.7], sucrose [0.25 mmol/L], dithiothreitol [0.5 mmol/L], aminohexanoic acid [1 mmol/L], and phenylmethylsulfonyl fluoride [1 mmol/L]). This was transferred to a 1-mL Dounce homogenizer (Fisher Scientific) and homogenized by 10 strokes (up and down while rotating the pestle). The homogenate was then transferred to the original microcentrifuge tube, which was centrifuged at 800*g* for 15 min. The supernatant was transferred to a new microcentrifuge tube, the pellet was washed with 100 μL of homogenization buffer, and the centrifugation was repeated. Total hexokinase activity was determined on a 50-μL sample. The remainder was centrifuged at 14,000*g* for 10 min in a microcentrifuge tube to pellet the mitochondria. The supernatant was saved, and the pellet was washed with 100 μL of homogenization buffer. The wash was added to the supernatant, and the hexokinase activity was determined on the combined "cytoplasmic" fraction. The mitochondria were resuspended in 70 μL of homogenization buffer and used to determine mitochondrial hexokinase activity.

Hexokinase was determined by adding 50 μL of homogenate to a cuvette containing Tris/HCl (100 mmol/L, pH 8.0), glucose (10 mmol/L), nicotinamide adenine dinucleotide phosphate ($NADP^+$) (0.4 mmol/L), $MgCl_2$ (10 mmol/L), ATP (5 mmol/L), and glucose-6-phosphate dehydrogenase (0.15 unit) and by following the production of reduced nicotinamide adenine dinucleotide phosphate ($NADP^+$) at 340 nm in a Helios γ-Series 2 ultraviolet-visible spectrometer (Thermo Spectronic; Fisher Scientific). Enzyme activity was expressed as mU/mg of protein using the extinction coefficient for reduced nicotinamide adenine dinucleotide phosphate of 6.3×10^3 mol^{-1} cm^{-1}.

Cytofluorimetric Analysis of Mitochondrial Potential and Annexin Binding

The membrane potential in MCF-7 cells was measured using the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red-to-green fluorescence-intensity ratio. The percentage of cells that emit only red fluorescence attributable to polarized mitochondrial membranes was determined. One flask of control cells was incubated for 30 min with medium containing the depolarizing compound valinomycin (1 μmol/L) (*22*). JC-1 was dissolved in dimethyl sulfoxide (5 mg/mL), and 50 μL were added to 10 mL of medium. Cells were incubated with 2 mL of medium containing JC-1 for 15 min at 37°C and then washed twice with PBS. Cells were then detached with trypsin and, after the addition of medium and centrifugation at 400*g* for 5 min, were resuspended in 0.5 mL of PBS. They were then analyzed on an LSR Research Flow Cytometer (Becton Dickinson) to detect green fluorescence at excitation/emission wavelengths of 485/530 nm and red fluorescence at excitation/emission wavelengths of 550/595 nm.

Apoptosis and cell viability were determined by annexin V-FITC binding to exposed phosphatidylserine and propidium iodide uptake using a kit (Sigma-Aldrich) and following the manufacturer's directions.

Determination of Cellular ATP Content and Lactate Production

Lactate was determined on medium sampled at the end of incubation with ^{18}F-FDG using a lactate assay kit (Biovision) and following the manufacturer's instructions. ATP was determined using a bioluminescent somatic cell assay kit (Sigma). Samples were prepared as described under "^{18}F-FDG Incorporation."

Statistics

Statistical differences between means were determined using the Student t test and were considered significant if P was less than 0.05.

RESULTS

Figure 1 shows the effect of 1 μmol of valinomycin per liter on JC-1 fluorescence: a loss of red fluorescence (loss of membrane potential–dependent accumulation in mitochondria) indicative of mitochondrial membrane depolarization. No increase was found in annexin binding or uptake of propidium iodide by cells treated with valinomycin for 40 min.

Treatment with valinomycin for 30 min, shown in Figure 2, resulted in significantly ($t = 5.97$, $P < 0.001$) increased ^{18}F-FDG incorporation by MCF-7 cells. Enhanced uptake was still apparent after 3.5 h of incubation ($t = 4.67$, $P < 0.01$). The initial rate of OMG transport, shown in Figure 3 (a measure of glucose transport), was significantly increased ($t = 3.27$, $P < 0.01$) by about 25% in cells treated for 30 min with valinomycin, compared with untreated cells. Figure 4 shows the effects of valinomycin treatment on total hexokinase activity and the percentage of mitochondrial bound hexokinase activity. These effects were not statistically significantly different from those in untreated cells (total activity: $t = 0.64$; mitochondrial activity fraction: $t = 1.24$).

Table 1 shows the cellular ATP content and rate of lactate production. ATP content in valinomycin-treated cells was similar to that in control cells ($t = 1.78$, not statistically significant), whereas lactate production was significantly increased ($t = 4.1$, $P < 0.001$) in cells treated for 30 min with valinomycin.

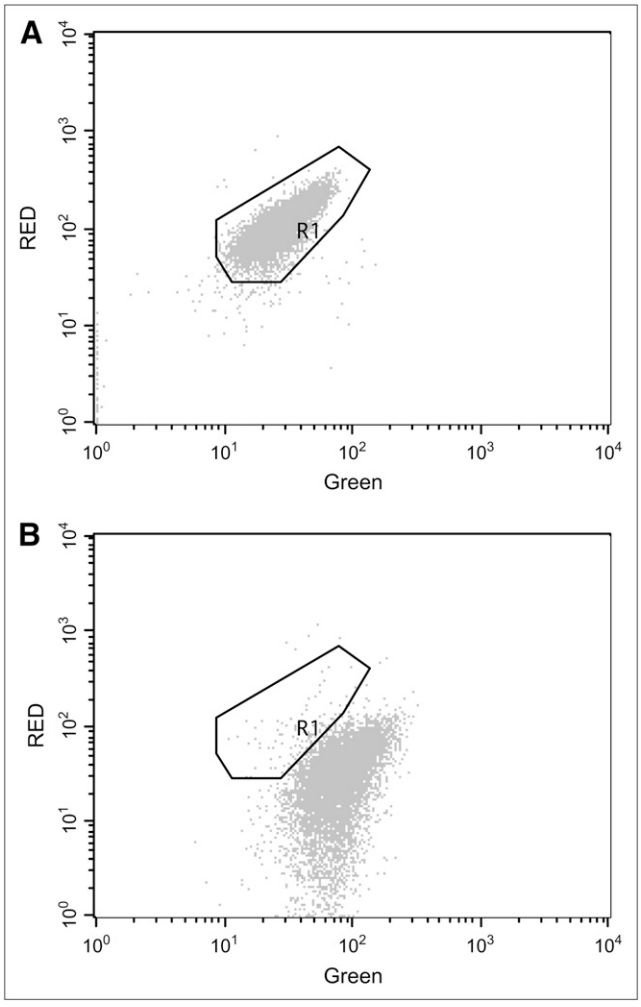

FIGURE 1. Fluorescence of MCF-7 control cells (A) and valinomycin-treated cells (30 min) (B) incubated with mitochondrial membrane potential probe JC-1. *x*- and *y*-axes are green and red fluorescence, respectively. Valinomycin-treated cells show loss of red fluorescence (loss of membrane potential–dependent accumulation in mitochondria) indicative of mitochondrial membrane depolarization.

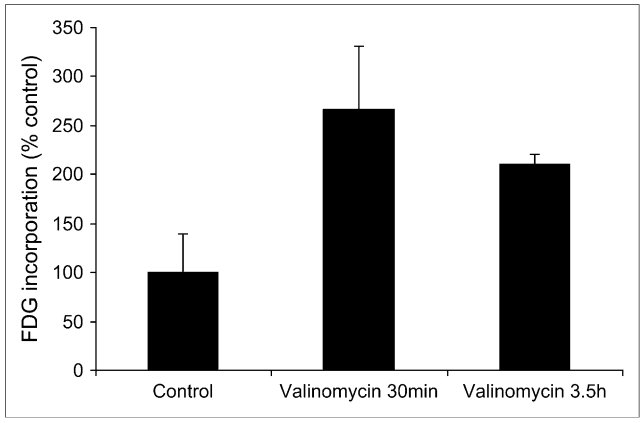

FIGURE 2. ^{18}F-FDG incorporation by control MCF-7 cells ($n = 7$) treated for 30 min ($n = 7$) and 3.5 h ($n = 4$) with valinomycin (% control).

DISCUSSION

^{18}F-FDG incorporation is generally found to be decreased, compared with pretreatment levels, in responding tumors when PET scans are performed a week or so after

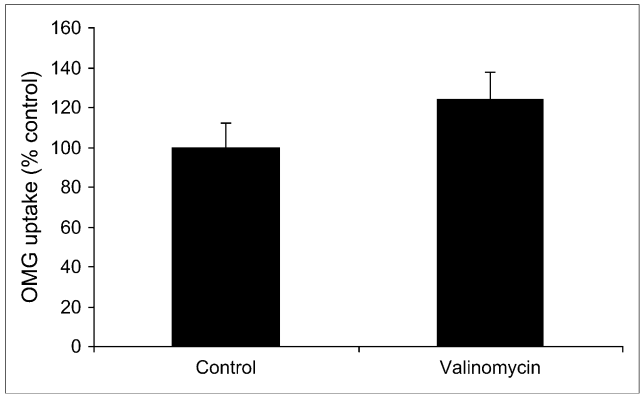

FIGURE 3. Initial rate of OMG uptake by cells treated for 30 min with valinomycin ($n = 7$, treated and controls).

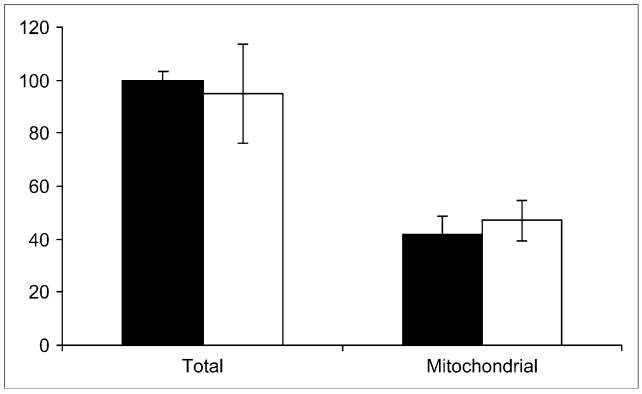

FIGURE 4. Hexokinase activity total (% control) and mitochondrial hexokinase (% total activity by cell extracts treated with valinomycin for 30 min) (n = 6, treated and control).

therapy. However, it has frequently been reported that increased ^{18}F-FDG incorporation shortly after therapy predicts a good response. De Witte et al. (*3*) determined ^{18}F-FDG uptake by gliomas before and 24 h after treatment with carmustine and found that a hypermetabolic reaction after therapy predicted patient survival. In a study of 8 patients with brain tumors, Maruyama et al. (*4*) showed increased ^{18}F-FDG uptake 4 h after treatment with a single radiation dose. Studies of cultured cells have also shown increased ^{18}F-FDG (or ^{3}H-deoxyglucose) uptake a few hours after treatment. Fujibayashi et al. (*6*) showed that treatment of LS180 human colon adenosarcoma cells with 3 or 30 Gy of radiation resulted in enhanced 2-deoxy-D-glucose uptake between 1 and 4 h after irradiation. Similarly, Haberkorn et al. (*7*) observed increased ^{18}F-FDG uptake by MCF-7 cells between 1 and 4 h after treatment with the apoptosis-inducing phospholipid hexadecylphosphocholine (*10*) and, in a later study (*8*), a dose-dependent increase in ^{18}F-FDG incorporation 4 h after treatment of rat prostate adenocarcinoma cells with the cytidine analog gemcitabine. Higashi et al. (*11*) treated human ovarian cancer cells with radiation and found increased ^{18}F-FDG incorporation between 0 and 12 d afterward.

The onset of apoptosis, a mechanism of cell death associated with response to radiation and chemotherapy, involves early loss of mitochondrial membrane potential (*14*). In this study, we found that ^{18}F-FDG incorporation was enhanced by depolarization of the mitochondrial membrane in MCF-7 cells by treatment with valinomycin for 30 min before phosphatidylserine was exposed on the cell surface. Lactate production was also increased, suggesting an increased rate of glycolysis. Several ^{1}H nuclear magnetic resonance studies have reported increased lactate production by tumors both in vivo and in vitro (*23–25*) during response to treatment. Santini et al. (*23*) showed increased lactate levels by osteosarcoma cells during the first 24 h after treatment with radiation. The posttherapy increase in lactate production occurred in cells grown both as monolayers and as 3-dimensional microspheroids. Lutz et al. (*24*) showed that treatment of human colon adenocarcinoma cells (HT-29) with a combination of γ-interferon and tumor necrosis factor α, which was shown to induce apoptosis, caused a time-dependent increase in lactate production between 4 and 15 h after the addition of treatment. Increased lactate levels have also been detected after treatment of HeLa tumor cells with radiation (*25*).

TABLE 1
ATP Content and Lactate Production by Control and Treated MCF-7 Cells

MCF-7 cell group	ATP*	Lactate†
Control	15.4 ± 4.6	3.4 ± 0.98
Valinomycin-treated (30 min)	19 ± 5.8	5.4 ± 0.95

*μg/mg of protein; n = 15 for both.
†μmol formed/mg of protein/15 min; n = 8 for both.

^{18}F-FDG incorporation can be modulated by changes in the rate of glucose transport, hexokinase activity, and the rate of dephosphorylation by glucose-6-phosphatase. However, MCF-7 cells do not express glucose-6-phosphatase (*19*). Further, hexokinase activity and its subcellular location were found not to be altered by treatment of cells with valinomycin for 30 min. Glucose transport was significantly increased—an effect that would increase the rate of ^{18}F-FDG uptake. However, the increase of glucose transport was only 25%, whereas the increase in ^{18}F-FDG incorporation was over 100%.

The availability of ATP (*26*) is associated with ^{18}F-FDG incorporation, but in the present study we did not find a significant change in cellular ATP content after treatment with valinomycin despite mitochondrial membrane potential's being crucial for the activity of ATP synthase (*18*). Previous studies have also shown that ATP content can be maintained or even enhanced after treatment with dissipaters of mitochondrial membrane (*15,17,18*) but not after inhibitors of glycolysis (*16*). Noda et al. (*27*) have shown that inhibition of oxidative phosphorylation results in a buildup of reduced nicotinamide adenine dinucleotide in the mitochondria and retrogressively into the cytoplasm, resulting in an enhanced rate of lactate formation (which requires reduced nicotinamide adenine dinucleotide). The increase in lactate production after treating cells with valinomycin, observed in the present study, suggests that ATP synthesis by oxidative phosphorylation is switched to anaerobic synthesis. This change would result in a more rapid glycolytic flux and more incorporation of ^{18}F-FDG. It is also possible that ATP synthesized in the cytoplasm by glycolysis, rather than by mitochondrial processes, is more readily available for phosphorylating of ^{18}F-FDG. However, this possibility contradicts the commonly held view that mitochondrial hexokinase activity using ATP produced in the mitochondria is the most important factor in ^{18}F-FDG incorporation (*22,28*).

CONCLUSION

Dissipation of the mitochondrial membrane potential of tumor cells by treatment with valinomycin enhances ^{18}F-FDG incorporation, glucose transport, and lactate production but does not affect ATP content, hexokinase activity, or hexokinase subcellular distribution.

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