Suppression of human tumor cell proliferation through mitochondrial targeting

EKHSON HOLMUHAMEDOV, LIONEL LEWIS,* MARTIN BIENENGRAEBER, MADINA HOLMUHAMEDOVA, ARSHAD JAHANGIR, AND ANDRE TERZIC

Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Mayo Foundation, Rochester, Minnesota, USA; and *Section of Clinical Pharmacology, Department of Medicine, Dartmouth Medical School and Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire, USA

ABSTRACT Intracellular calcium signaling plays a central role in cell proliferation. In leukemic cells, the calcium release-activated calcium channels provide a major pathway for calcium entry ($I_{\text{CRAC}}$) perpetuating progression through the cell cycle. Although $I_{\text{CRAC}}$ is under mitochondrial regulation, targeting mitochondrial function has not been exploited to control malignant cell growth. The benzothiadiazine diazoxide, which depolarized respiration-dependent mitochondrial membrane potential, reduced the rate of proliferation and arrested human acute leukemic T cells in the G0/G1 phase. Diazoxide did not alter cellular energetics, but rather inhibited the mitochondria-controlled $I_{\text{CRAC}}$ and reduced calcium influx into tumor cells. The antiproliferative action of diazoxide was mimicked by removal of extracellular calcium or by the tyrphostin A9, an $I_{\text{CRAC}}$ inhibitor. Deletion of the mitochondrial genome, which encodes essential respiratory chain enzyme subunits, attenuated the inhibitory effect of diazoxide on $I_{\text{CRAC}}$-mediated calcium influx and cell proliferation. Thus, manipulation of mitochondrial function and associated calcium signaling provides a basis for a novel anticancer strategy.—Holmuhamedov, E., Lewis, L., Bienengraeber, M., Holmuhamedova, M., Jahangir, A., Terzic, A. Suppression of human tumor cell proliferation through mitochondrial targeting. FASEB J. 16, 1010–1016 (2002)

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Proliferation of leukemic cells is under control of intracellular Ca$^{2+}$ signaling (1). Prolonged elevation of cytosolic Ca$^{2+}$ regulates nucleo-cytoplasmic communication, increases the efficiency and specificity of gene transcription, and drives cells through Ca$^{2+}$-sensitive checkpoints of the cell cycle (2–6). It has become increasingly evident that mitochondria play a central role in shaping the pattern of intracellular Ca$^{2+}$ dynamics (7–9). In T lymphocytic leukemia cells, gating of the Ca$^{2+}$ release-activated Ca$^{2+}$ channels (CRAC), as well as the rate and magnitude of Ca$^{2+}$ influx ($I_{\text{CRAC}}$) through these store-operated Ca$^{2+}$ channels, are determined by the mitochondrial status, underscoring the significance of mitochondria in defining cell cycle progression (10–16).

A potential dependent Ca$^{2+}$ uptake into energized mitochondria reduces cytosolic Ca$^{2+}$ in the proximity of CRAC channels, promoting channel opening (11, 13, 15). Conversely, in cells with de-energized mitochondria and reduced mitochondrial Ca$^{2+}$ uptake, the elevated free Ca$^{2+}$ concentration maintains store-operated channels closed in accord with the Ca$^{2+}$-dependent inactivation of $I_{\text{CRAC}}$ (14–16). Although mitochondria have the potential to regulate intracellular Ca$^{2+}$ signaling and determine the transcriptional potential of a cell (15), targeting mitochondria has not been exploited to control $I_{\text{CRAC}}$-mediated Ca$^{2+}$ influx and associated cell proliferation.

Prototypic drugs such as diazoxide, which display a predilection for the inner mitochondrial membrane, were recently recognized for their ability to oxidize components of the respiratory chain and reduce mitochondrial membrane potential (17–19). We demonstrate that diazoxide suppresses proliferation of human acute T lymphocytic leukemia cells through mitochondrial targeting. Diazoxide depolarized mitochondria and inhibited mitochondria-dependent Ca$^{2+}$ influx, arresting cell cycle progression without disturbing cell energetics. The antiproliferative action of diazoxide was lost in cells with deleted mitochondrial DNA lacking functional mitochondria. These findings indicate that manipulation of functional mitochondria is an effective strategy of disrupting intracellular Ca$^{2+}$ signaling and cell proliferation.

MATERIALS AND METHODS

Tumor cells with and without mitochondrial DNA

Human T leukemic Jurkat and Molt-4 cell lines (American Type Culture Collection, Rockville, MD) were cultured at an initial density of 5×10$^4$ cells/mL in RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (37°C, 5% CO$_2$/95% air). To obtain
TARGETING MITOCHONDRIA FOR TUMOR SUPPRESSION

RESULTS

Suppression of cell proliferation by diazoxide

Human leukemic Jurkat cells proliferated vigorously at a rate of 7600 ± 840 cells/h (n=10; Fig. 1A, B). Typical for asynchronous growth, Jurkat cells displayed a broad cell cycle distribution, with 52 ± 3%, 32 ± 2% and 17 ± 3% (n=9) of the cell population in the G0/G1, S and G2/M phase, respectively (Fig. 1C, inset). Diazoxide (100 μM) dramatically reduced the proliferation rate to 2650 ± 330 cells/h (n=10), resulting in a threefold decline in growth compared to untreated cells (P<0.01; Fig. 1A, B). In fact, diazoxide (100 μM) arrested Jurkat cells in the G0/G1 phase away from the S phase (Fig. 1C). On average, diazoxide increased the percentage of cells in the G0/G1 phase to 71 ± 2% (n=9), a 36% increase compared to untreated cells (P<0.01). In parallel, diazoxide decreased the number

with an EM-1200 EX II electron microscope (Jeol, Peabody, MA) as described (23, 24).

Cellular respiration and ATP content

The rate of oxygen consumption was determined with an oxygen-sensitive electrode at 37°C in cells incubated (in mM: KCl 110, NaCl 10, KH2PO4 2, MgSO4 1, pyruvate 5, malate 5, HEPES 20, pH 7.15) in the presence of saponin (0.005%) to provide access for mitochondrial substrates (19). Cellular ATP was determined by high-pressure liquid chromatography (Hewlett Packard, Palo Alto, CA) of the acid-soluble cellular extract neutralized with K2CO3:HEPES (2.5 M/1 M) as described (23, 25).

Isolated mitochondria

Mitochondria were isolated after cell homogenization with a Dounce homogenizer in buffer containing (in mM: mannitol 220, sucrose 50, MOPS 10, PMSF 1, and EGTA 1, pH 7.3) using differential centrifugation (26). Mitochondria washed once with isolation buffer (without EGTA) were used immediately or stored with 1 mM PMSF/1 mM DTT in liquid nitrogen until processed. Mitochondrial cytochrome c and citrate synthase activities were measured from the oxidation of cytochrome c (550 nm) and deacetylation of acetyl-CoA (412 nm) using a DU-7400 spectrophotometer (26).

Experimental compounds

Diazoxide, thapsigargin, carboxyl cyanide m-chlorophenylhydrazone (CCCP), and the tyrphostin A9 (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide (Sigma). The maximal concentration of solvent in the incubation medium was under 0.5%.

Statistical analysis

Data are expressed as mean ± se. Where appropriate, Student’s t test or analysis of variance was performed for comparison between groups using a statistical analysis software (JMP, SAS Institute, Cary, NC). A value of P < 0.01 was considered statistically significant.

Cell cycle distribution

Cell number was determined using a hemocytometer (Bright Line, Hauser Scientific, Horsham, PA), and cell cycle distribution analyzed with a fluorescent activated cell sorter (Vantage Cytofluorimeter, Becton Dickinson, Rutherford, NJ). For flow cytometry, cells fixed in 70% ethanol and washed with Ca2+ /Mg2+-free phosphate buffer were treated with Rnase, and nuclear DNA was labeled with 50 μg/μL propidium iodide. After data acquisition, the CellQuest software (Becton Dickinson, Rutherford, NJ) was used to analyze nuclear DNA distribution (22).

Confocal microscopy and spectrofluorometry

Cells were labeled with mitochondria-specific fluorescent dyes Mito-Tracker Green-FM and 10-nonyl acridine Orange (Molecular Probes), and the intracellular distribution of mitochondria was examined by laser scanning confocal microscopy (LSM 510, Carl Zeiss) as described (9, 19). Cytoplasmic Ca2+ was imaged in separate experiments in cells loaded with Fluo-3/AM, and intracellular Ca2+ levels determined off-line using the image analysis software ANALYZE (Mayo Clinic, Rochester, MN). The rate of Ca2+ influx was determined from changes in the concentration of intracellular Ca2+ monitored by Indo-1 (Molecular Probes) (16), using a spectrofluorometer (SPEX Fluorolog, SPEX Industry).

Electron microscopy

Cells (~2-106) were fixed using Trump’s buffer (1% glutaraldehyde, 4% formaldehyde, 0.1 M phosphate buffer, pH 7.2), rinsed, and postfixed in phosphate-buffered 1% osmium tetroxide. Samples were stained en bloc with 2% uranyl acetate for 30 min at 60°C, rinsed, dehydrated, and embedded in Spurr’s resin. Thin sections were cut on an Ultracut E ultramicrotome (Reichert-Jung, Wien, Austria), placed on copper grids, stained with lead citrate, and micrographed with an EM-1200 EX II electron microscope (Jeol, Peabody, MA) as described (23, 24).

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of cells in the S phase to 20 ± 2% \((n=9)\), a 38% reduction \(P<0.01; \) Fig. 1C), without significantly affecting the G2/M phase \((n=9; \) Fig. 1C). Upon removal of diazoxide, Jurkat cells resumed their initial rate of proliferation and continued to grow at 7920 ± 880 cells/h, a value not significantly different from that of untreated cells \(P=0.17; \) Fig. 1A, B). Thus, diazoxide suppressed proliferation of acute leukemic T cells associated with reversible inhibition of cell growth.

**Targeting mitochondria reduces cellular Ca\(^{2+}\) influx required for proliferation**

Growth and cell cycle progress depend on intracellular energy supply and calcium signaling \(1\). In leukemic cells, mitochondria are central in both processes by providing ATP through oxidative phosphorylation and by regulating store-operated Ca\(^{2+}\) influx \(I_{\text{CRAC}}\) through membrane potential-dependent Ca\(^{2+}\) buffering \(1, 12–16\). Proliferating Jurkat cells demonstrated abundance of energized mitochondria distributed throughout the cytosol \(\text{Fig. } 2A, B\), with a capability to avidly consume oxygen \(\text{Fig. } 2C\). Diazoxide \(100 \mu M\) significantly increased the rate of oxygen consumption \(\text{Fig. } 2C, D\) and depolarized mitochondrial membrane \(\text{Fig. } 2E, F\). Intracellular ATP concentration was, however, maintained at 1.48 ± 0.11 and 1.29 ± 0.09 nmol ATP/mg protein \(n=3; P=0.15\) in the absence and presence of diazoxide \(100 \mu M\), indicating that diazoxide-induced mitochondrial depolarization was insufficient to interrupt cellular energy metabolism \(\text{Fig. 3A}\). Yet diazoxide significantly reduced cytosolic Ca\(^{2+}\), by 66 ± 7%, from 30854 ± 5287 to 10365 ± 2213 pixels of Fluo-3 fluorescence per image in untreated vs. diazoxide-treated cells \(n=27; \) Fig. 3B). In lymphocytic T cells, intracellular Ca\(^{2+}\) refills primarily through \(I_{\text{CRAC}}\) the mitochondria-dependent store-operated Ca\(^{2+}\) influx channel \(12, 13, 16\). In Jurkat cells with intact mitochondria, depletion of intracellular Ca\(^{2+}\) stores with thapsigargin \(1 \mu M\), a blocker of the endoplasmic reticulum Ca\(^{2+}\)-ATPase \(16\) promoted vigorous Ca\(^{2+}\) influx \(\text{Fig. 3C}\) sensitive to the tyrphostin A9 \(1 \mu M\),
Diazoxide reduces cytosolic Ca$^{2+}$ and inhibits I$_{CRAC}$ required for proliferation of Jurkat cells. A) Intracellular ATP levels are maintained in the presence of diazoxide (100 μM), which B) significantly reduced cytosolic Ca$^{2+}$. A total of 2-10$^6$ cells were used for the ATP assay, while Ca$^{2+}$ was monitored in Fluo-3 AM loaded cells using scanning confocal microscopy. C) Emptying of intracellular Ca$^{2+}$ stores with thapsigargin (1 μM) in Ca$^{2+}$-free medium, activates Ca$^{2+}$ influx through I$_{CRAC}$ monitored in 5 μM Indo-1 AM loaded cells on addition of 3 mM of extracellular CaCl$_2$ (arrow). Ca$^{2+}$ influx is inhibited by 1 μM A9, an inhibitor of I$_{CRAC}$ or 0.1 μM CCCP, an uncoupler of mitochondrial oxidative phosphorylation, and reduced by diazoxide (100 μM). D) Cell growth was obtained in the presence of 335 μM Ca(NO$_3$)$_2$ (open bar), and was inhibited by removal of Ca$^{2+}$ (closed bar) or by addition of 1 μM A9 (hatched bar). Asterisk indicates significant difference with control.

Mitochondrial biogenesis critically depends on mitochondrial DNA-encoded polypeptides of the respiratory chain (20, 21, 28). To determine the contribution of mitochondria in the antiproliferative effect of diazoxide, cells void of functional mitochondria (rho$^0$) were generated by depletion of mitochondrial DNA with ethidium bromide, a potent inhibitor of mitochondrial DNA replication and transcription (20, 21, 29). The parental malignant lymphoid cell line Molt-4 (WT) possessed intact mitochondrial membranes and typical crista, a signature of functional mitochondria (Fig. 4A and inset). In contrast, rho$^0$ cells displayed an aberrant mitochondrial phenotype ('mitochondrial ghost') with grossly distorted cristae and essentially an empty matrix (Fig. 4B and inset). Loss of mitochondrial DNA in rho$^0$ cells, confirmed by quantitative PCR amplification (Fig. 4C), was associated with reduced expression of the

**Figure 3.** Diazoxide reduces cytosolic Ca$^{2+}$ and inhibits I$_{CRAC}$ required for proliferation of Jurkat cells. A) Intracellular ATP levels are maintained in the presence of diazoxide (100 μM), which B) significantly reduced cytosolic Ca$^{2+}$. A total of 2-10$^6$ cells were used for the ATP assay, while Ca$^{2+}$ was monitored in Fluo-3 AM loaded cells using scanning confocal microscopy. C) Emptying of intracellular Ca$^{2+}$ stores with thapsigargin (1 μM) in Ca$^{2+}$-free medium, activates Ca$^{2+}$ influx through I$_{CRAC}$ monitored in 5 μM Indo-1 AM loaded cells on addition of 3 mM of extracellular CaCl$_2$ (arrow). Ca$^{2+}$ influx is inhibited by 1 μM A9, an inhibitor of I$_{CRAC}$ or 0.1 μM CCCP, an uncoupler of mitochondrial oxidative phosphorylation, and reduced by diazoxide (100 μM). D) Cell growth was obtained in the presence of 335 μM Ca(NO$_3$)$_2$ (open bar), and was inhibited by removal of Ca$^{2+}$ (closed bar) or by addition of 1 μM A9 (hatched bar). Asterisk indicates significant difference with control.

**Figure 4.** Mitochondrial structure and function in wild-type parental Molt-4 and mitochondrial DNA-depleted rho$^0$ cells. A) Electron micrographs of a wild-type parental (WT) T lymphocytic Molt-4 cell with typical intramitochondrial organization, dense matrix and abundant cristae (A, inset). B) A mitochondrial DNA-devoid rho$^0$ cell with a 'mitochondrial ghost' appearance due to absence of electron-dense mitochondrial matrix and disorganized cristae (B, inset). C) Mitochondrial DNA (mtDNA) content in parental Molt-4 (WT) and rho$^0$ cells measured by quantitative PCR (upper panel). Average mitochondrial DNA content in WT (open bar) and rho$^0$ (closed bar) cells (lower panel). D) Southern blot analysis (upper panel) and activity (lower panel) of the mitochondrial genome-encoded subunit II of cytochrome c oxidase (COX II) in parental (WT) and mtDNA-deficient rho$^0$ cells. C, D) Asterisk indicates significant difference between parental and mtDNA-deficient cells. E) Respective Southern blot analysis and activity of the nuclear genome encoded β-actin (upper panel) and citrate synthase (lower panel) in parental (WT) and mtDNA-deficient (rho$^0$) cells. F) Reduced cytochrome c oxidation in mtDNA-deficient (rho$^0$) vs. parental (WT) cells.
mitochondrial gene-encoded subunit II of cytochrome c oxidase (Fig. 4D, upper panel). The activity of cytochrome c oxidase was 25 ± 3 vs. 3 ± 1 nmol cytochrome c/min/10⁶ cells in Molt-4 and rho⁰ cells, respectively (n=4, P<0.01; Fig. 4D, lower panel, F). Rhö⁰ cells did, however, maintain an intact nuclear genome, with comparable expression of the β-actin gene in wild-type and mitochondrial DNA-deficient cells (Fig. 4E, upper panel) and unchanged activity of citrate synthase, a marker of a mitochondrial enzyme encoded by nuclear DNA (Fig. 4E, lower panel). Loss of functional mitochondria resulted in defective responsiveness of rho⁰ cells to diazoxide (Fig. 5). Whereas diazoxide (100 μM) essentially halved cytoplasmic Ca²⁺ concentration in parental Molt-4 cells (Fig. 5A, top panel), the mitochondrial potassium channel opener did not significantly modulate Ca²⁺ levels in rho⁰ cells (Fig. 5A, lower panel). Indeed, the diazoxide-induced reduction in Ca²⁺ concentration was 41 ± 3% vs. 1 ± 1%, respectively (P<0.01). Diazoxide was a less effective inhibitor of store-operated Ca²⁺ influx in mitochondrial-deficient compared to parental cells (Fig. 5B). On average, diazoxide (100 μM) reduced the rate of thapsigargin-activated Ca²⁺ influx into wild-type parental cells from 8.5 ± 0.5 to 5.9 ± 0.9 RFU/min/10⁶ cells, a 41 ± 3% inhibition of Ca²⁺ influx (n=6, P<0.01). In contrast, in rho⁰ cells, diazoxide did not significantly affect the rates of Ca²⁺ influx, which were 3.2 ± 0.2 and 2.9 ± 0.3 RFU/min/10⁶ cells in the absence and presence of the drug (n=6; P=0.25). Accordingly, in parental cells, diazoxide (100 μM) impaired the rate of proliferation, by 43 ± 5% (n=3), but this antiproliferative effect was significantly attenuated (15±6%, n=3) in mitochondrial DNA-deficient rho⁰ cells (Fig. 5C, P<0.01). Thus, deletion of the mitochondrial genome, encoding essential enzyme subunits of the respiratory chain, reduced diazoxide-mediated inhibition of store-operated calcium influx and tumor cell growth.

**DISCUSSION**

Traditionally, mitochondria have been recognized as the main source of intracellular energy production securing energetic homeostasis of the cell through oxidative phosphorylation (30). More recently it has become apparent that mitochondria are also central in cellular Ca²⁺ signaling and contribute to intracellular Ca²⁺ dynamics by virtue of their membrane potential-dependent Ca²⁺ buffering capacity (1, 7–9, 13) and proximity to specialized cellular Ca²⁺ compartments (31). Mitochondria are critical in the regulation of vital Ca²⁺-dependent processes, including activation of transcription factors and gene expression (3, 5). Commitment to and completion of the cell division cycle in cells with a high metabolic drive, such as fast growing cancer cells, are processes particularly sensitive to inhibition of mitochondrial function (32). Here, we exploited mitochondrial control over intracellular Ca²⁺ and demonstrate that depolarization of mitochondrial membrane potential down-regulates intracellular Ca²⁺, suppressing proliferation in human leukemia cell lines. Thus, targeting mitochondria to disrupt communication between mitochondria and Ca²⁺ signaling pathways emerges as an efficient approach in control of malignant cell growth.

In human leukemia cells such as T lymphocyte-derived Jurkat and Molt-4 cells used here, the mitochondrial functional status determines Ca²⁺-dependent gating of the store-operated Ca²⁺ current I₉⁵ Venus a key pathway for sustained Ca²⁺ entry (1, 13). High mitochondrial membrane potential promotes mitochondrial Ca²⁺ uptake and activates I₉⁵ Venus leading to transcriptional activation and progression through cell cycle (15, 32). We demonstrate that diazoxide, which targets the inner mitochondrial membrane and oxidizes the respiratory chain (17, 18), inhibited I₉⁵ Venus and suppressed cell proliferation. The effects of diazoxide on Ca²⁺ flux and cell proliferation were attenuated in respiratory-deficient mitochondrial DNA-depleted cells,
underscoring the requirement for a functional electron transport chain in mediating the action of diazoxide. Previous reports identify mitochondrial succinate dehydrogenase (33, 34) and a mitochondrial K⁺ conductance (17, 18, 35) as molecular targets for diazoxide. Indeed, inhibition of a key component in the respiratory chain and/or activation of potassium influx would result in mitochondrial depolarization (36, 37), in accord with the reduction in membrane potential induced here by diazoxide. A surge in mitochondrial membrane potential during the G0/G1 phase of the cell cycle is a prerequisite for proliferating cells to engage into the S phase (32). Clamping mitochondrial membrane potential at a depolarized level by diazoxide provides a mechanistic basis for the antiproliferative action and cell arrest in the G0/G1 phase away from the S phase. Such a property could be explored to develop novel adjunctive therapy in the treatment of proliferative disorders.

In summary, this study demonstrates a novel antiproliferative mechanism through regulation of mitochondria-dependent intracellular Ca²⁺ homeostasis. Specifically, we show suppression of leukemic cell proliferation by the benzothiadiazine diazoxide through depolarization of the mitochondrial membrane and disruption of intracellular Ca²⁺ dynamics. Deletion of mitochondrial DNA attenuated the antiproliferative action of diazoxide, underscoring the critical role of functional mitochondria and associated Ca²⁺ signaling in cell cycle progression. In this way, mitochondria by orchestrating intracellular Ca²⁺ homeostasis emerge as unique targets for regulation of malignant growth and proliferation.

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