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Extracellular ATP is internalized by macropinocytosis and induces intracellular ATP increase and drug resistance in cancer cells

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ATP plays central roles in cancer metabolism and the Warburg effect. Intratumoral ATP concentrations are up to 10^4 times higher than those of interstitial ATP in normal tissues. However, extracellular ATP is not known to enter cancer cells. Here we report that human A549 lung cancer cells internalized extracellular ATP by macropinocytosis as demonstrated by colocalization of a nonhydrolyzable fluorescent ATP and a macropinocytosis tracer high-molecular-weight dextran, as well as by a macropinocytosis inhibitor study. Extracellular ATP also induced increase of intracellular ATP levels, without involving transcription and translation at significant levels, and cancer cells' resistance to ATP-competitor anticancer drugs, likely through the mechanism of ATP internalization. These findings, described for the first time, have profound implications in ATP-sharing among cancer cells in tumors and highlight a novel anticancer target.

Introduction

Metabolic reprogramming, or deregulation of cellular energetics, is now recognized as a hallmark of cancer [1]. ATP production by highly upregulated glycolysis in cancer cells even when oxygen is abundant, a phenomenon known as the Warburg effect [2,3], is an area of intensive investigation with improved but still incomplete understanding. Interpretations of the functional reasons for upregulated glycolysis in the presence of continued mitochondrial oxidative phosphorylation (OXPHOS) and the relationship between glycolytic synthesis of ATP and other metabolic intermediates in cancer cells are controversial and evolving [3–6]. Being metabolically heterogeneous, some cancer cells in tumors make significantly less ATP than other cancer cells or even normoxic normal cells [3]. However, cancer cells appear to be able to obtain all the ATP they need regardless of oxygen status. This is puzzling unless previously unrecognized mechanisms for securing ATP exist.

One potential source of ATP for cancer cells is the extracellular ATP pool [7]. Interstitial ATP concentrations in normal tissues are in the range of 1–1000 nM [8,9]. In contrast, intratumoral ATP levels, i.e., extracellular ATP levels inside tumors, have recently been measured in the range of several hundred μM or higher [10–13], or 10^3–10^4 times of those in normal tissues. However, neither the source of the ATP nor the destination of the molecule is known. Earlier experimental evidence suggests uptake [14–18] and release of ATP in normal animal cells [19,20], providing conceptual and biological basis for potential ATP uptake by cancer cells. However, the uptake of extracellular ATP by cancer cells has never been demonstrated except by artificial means [21].

In our recent anticancer therapeutic studies, we observed that extracellular ATP significantly increased intracellular ATP levels in A549 human lung cancer cells and rescued glucose-deprived cancer cells treated with glucose transporter 1 (GLUT1) inhibitor WZB117 [22]. However, extracellular ATP did not rescue A549 cells treated with paclitaxel, a drug with an ATP-independent anticancer mechanism [22]. One of the possible explanations for these results is that extracellular ATP is directly taken up by A549 cells and contributes to intracellular ATP concentration increase, playing a significant role in cancer cell growth and survival. Because ATP is

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charged and therefore hydrophilic, it cannot cross the plasma membrane by itself. However, no plasma membrane-associated ATP transporter has ever been found. Thus, we further speculated that the ATP uptake might be mediated by some types of endocytic processes, bypassing the problem. In the present study, we tested this hypothesis by studying ATP transport mechanisms with a nonhydrolyzable fluorescent ATP. Extracellular ATP concentrations in the reported intratumoral ATP range were used to mimic *in vivo* conditions. To identify the functional significance of the extracellular ATP-induced intracellular ATP increase, extracellular ATP-induced drug resistance was also studied. The use of a nonhydrolyzable fluorescent ATP for demonstrating ATP internalization and extracellular ATP-mediated drug resistance in cancer cells have never been reported. The findings of this study may significantly impact our interpretation of the Warburg effect, expand our knowledge of ATP/energy sharing among cancer cells, and highlight a new target for cancer treatment.

Materials and methods

Compounds and cell lines

Glut1 inhibitor WZB117 was used as previously described [22,23]. ATP, oligomycin, compound C, cycloheximide, sunitinib, paclitaxel, ethyl isopropyl amiloride (EIPA), were from Sigma–Aldrich. Actinomycin D and pazopanib were from Calbiochem and LC labs, respectively. Human non-small cell lung cancer A549, human breast carcinoma MCF7, and their respective human nontumorigenic NL-20 lung cells and MCF12A breast cells, and RKO human colon cancer cells were from ATCC. All these cells were maintained and propagated using ATCC recommended cell culture media and conditions.

ATP rescue study and intracellular ATP measurement

The cell rescue study was conducted as previously described [22]. Intracellular ATP concentration was measured using an ATP-luciferase-based ATPlite luminescence ATP detection system (Perkin Elmer) by following the assay instructions. Briefly, cells in 96-well non-transparent plates were treated under different conditions. After treatment and media removal, wash, and cell lysis, intracellular ATP levels were measured using a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). The relative ATP concentration in mock-treated controls was assigned a value of 100%. Final relative ATP concentrations of the treated samples were calculated by dividing their ATP levels with those of the controls after all the samples were normalized by total protein. Methods for determining absolute intracellular ATP concentrations have not been developed and were unnecessary for this study.

Protein analysis, clonogenic and flow cytometry assays of cancer cells

Western blot analyses were performed using the standard protocol. Antibodies against phosphorylated and total AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), eIF-2α, and β-actin were from Cell Signaling. β-actin was used as the protein loading control.

Clonogenic assay and flow cytometry analysis of differentially treated A549 cancer cells were performed as described previously [22]. Cells were treated with 100 nM oligomycin and DMEM supplemented with 2 mM glucose (glucose deprivation) with or without 3 mM ATP for 24 h before assays.

Dose- and time-dependence studies of ATP

For dose-dependence studies, ATP concentrations in the reported intratumoral ATP concentration range [10–13] were specifically chosen and used to treat cancer and nontumorigenic cells for 24 h. For time-dependence studies, 3 mM ATP-containing cell culture media were used to treat cancer cells for different durations. After treatment, cells were washed, lysed, and their intracellular ATP measured.

Fluorescence microscopy and ATP localization studies

Fluorescence microscopy was performed as previously described [24]. Briefly, A549 or MCF7 cells were seeded onto glass coverslips. Twenty-four hours after seeding, cells were serum-starved for 15 h. High-molecular-weight (70 kDa) fluorescent TMR-dextran (Invitrogen), a tracer for visualizing macropinosomes, was added to serum-free medium at a final concentration of 1 mg/mL for 30 min at 37 °C. After incubation, cells were rinsed five times in PBS and fixed in 3.7% formaldehyde for 15 min. Coverslips were mounted onto slides using Gold Antifade Reagent with DAPI (Invitrogen). Images were captured using epi-fluorescence microscope (ECLIPSE E600, Nikon), and analyzed using ImageJ (National Institutes of Health). For the colocalization study, both TMR-dextran and nonhydrolyzable fluorescent ATP (Jena Bioscience, Germany) were added to serum-free medium at a final concentration of 1 mg/ml and 10 μM, respectively. The assay was performed the same way as described for dextran.

Oligomycin and glucose deprivation assays – time course study

Endogenous ATP synthesis in cancer cells was inhibited by either glucose deprivation (GD) that reduces glycolysis or by oligomycin that blocks OXPHOS in the presence or absence of extracellular ATP. Cell culture media with reduced glucose concentrations (0.5–2 mM or 2–8% of the glucose concentration in the high glucose cell culture medium) were prepared as previously described [22]. Oligomycin at 0.25 or 0.5 μM was used to block OXPHOS for 2–24 h. After treatments, intracellular ATP levels in the treated cells were measured.

Studies with inhibitors of AMPK, transcription, translation

To determine the role of AMPK, gene expression, and protein synthesis in the intracellular ATP increase, AMPK inhibitor compound C [25,26], transcription inhibitor actinomycin D or translation inhibitor cycloheximide was individually added to A549 cells treated with oligomycin in the presence or absence of extracellular ATP for various times. After treatment, intracellular ATP levels were measured. Cells treated with oligomycin but with or without extracellular ATP were used as positive or negative controls.

Macropinocytosis inhibitor study

A549 cells grown in non-transparent black 96-well plates were treated with or without macropinocytosis inhibitor EIPA in the presence or absence of 1 mM extracellular ATP for various times. After treatment, cells were washed, lysed, and their intracellular ATP measured.

Drug resistance study

Tyrosine kinase inhibitors (TKIs) sunitinib [27] and pazopanib [28] were used to individually treat A549 cells grown in 96-well plates at various concentrations in the presence or absence of extracellular ATP with or without macropinocytosis inhibitor EIPA for 24 h. After treatment, cell viability of differentially treated cells was measured by the MTT assay. A non-TKI drug paclitaxel [29] was used as a control.

Experimental designs and statistical analysis

Each experimental condition was performed in triplicate and repeated at least once. Data were reported as mean ± standard deviation and analyzed using one-way ANOVA. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$.

Results

Extracellular ATP rescued cancer cells under different metabolic stresses and reduced anticancer efficacy of tyrosine kinase inhibitors

First, extracellular ATP-induced intracellular ATP increase and cancer cell rescue were investigated. When ATP was added to A549 cells treated with Glut1 inhibitor WZB117 [22], the cells were rescued from cell death in a dose-dependent manner (Fig. 1A). The treated cells displayed significantly elevated intracellular ATP levels (Fig. 1B). When A549 cells were treated with the OXPHOS inhibitor oligomycin in the presence of extracellular ATP, ATP reversed the upregulation of phosphorylation of metabolic stress marker eIF-2α [30] 24 h after the treatment (Fig. 1C), indicating reduced cell stress. Flow cytometry analysis revealed that addition of extracellular ATP enhanced viability of A549 cells, treated with oligomycin and glucose deprivation for 24 h, from ~39% to ~65% (Fig. 1D, bottom left quadrant, $P < 0.001$). The increased cell survival was further confirmed by a clonogenic assay (Fig. 1E, $P < 0.001$).

From these results, we speculated that the increased intracellular ATP might interfere with drugs that compete with ATP for their anticancer activity. Cell viability studies revealed that extracellular ATP at either 1 mM or 3 mM significantly reduced the anticancer