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The ATP/DNA Ratio Is a Better Indicator of Islet Cell Viability Than the ADP/ATP Ratio

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Abstract

Real-time, accurate assessment of islet viability is critical for avoiding transplantation of nontherapeutic preparations. Measurements of the intracellular ADP/ATP ratio have been recently proposed as useful prospective estimates of islet cell viability and potency. However, dead cells may be rapidly depleted of both ATP and ADP, which would render the ratio incapable of accounting for dead cells. Since the DNA of dead cells is expected to remain stable over prolonged periods of time (days), we hypothesized that use of the ATP/DNA ratio would take into account dead cells and may be a better indicator of islet cell viability than the ADP/ATP ratio. We tested this hypothesis using mixtures of healthy and lethally heat-treated (HT) rat insulinoma cells and human islets. Measurements of ATP/DNA and ADP/ATP from the known mixtures of healthy and HT cells and islets were used to evaluate how well these parameters correlated with viability. The results indicated that ATP and ADP were rapidly (within 1 hour) depleted in HT cells. The fraction of HT cells in a mixture correlated linearly with the ATP/DNA ratio, whereas the ADP/ADP ratio was highly scattered, remaining effectively unchanged. Despite similar limitations in both ADP/ADP and ATP/DNA ratios, in that ATP levels may fluctuate significantly and reversibly with metabolic stress, the results indicated that ATP/DNA was a better measure of islet viability than the ADP/ATP ratio.

Islet cell transplantation is emerging as a promising therapy for the treatment of type 1 diabetes.^{1–5} Despite recent advances, the transplantation of islets poses a unique challenge with respect to achieving a consistent clinical outcome. Part of this challenge is being able to reliably and rapidly assess clinical islet quality through the quantification of viability and function before transplantation. Current viability assays are limited in their ability to accurately predict transplantation outcomes in vivo.⁶ Consequently, to improve the clinical islet transplantation outcomes, it is imperative to develop more accurate viability assays.

A proposed method to assess islet viability and potency before transplantation is quantification of the ADP/ATP ratio.^{6–10} This ratio has been specifically applied in discriminating islet preparations that are suitable for clinical transplantation from those that are not.⁶ However, this application may be problematic under certain conditions. Intracellular ADP and ATP levels fluctuate rapidly because these high-energy phosphate molecules are rapidly produced and consumed in many intracellular biochemical reactions. It is widely known that viable cells turn over entire ATP stores on the order of minutes, and that dead cells are incapable of replenishing

their ATP stores when depleted.^{11,12} Furthermore, dead cells are rapidly depleted of their ADP and ATP.^{6,8,9,13} If dead cells are rapidly depleted of their ADP and ATP content, then the ADP/ATP ratio would be incapable of accounting for any dead cells found within an islet preparation and would be effectively overestimating the viability of the preparation.

To better account for dead cells and to more accurately assess the viability of an islet preparation, we suggest the use of an ATP/DNA ratio. DNA does not degrade as rapidly as ADP or ATP in a dead cell. Therefore, using direct measurements of DNA, dead cells in an islet preparation can be accounted for. Herein, we have described how the ATP/DNA ratio correlates more accurately with viability than the ADP/ATP ratio in mixtures of varying, known proportions of healthy/dead insulinoma (INS-1) cells and human islets.

MATERIALS AND METHODS

INS-1 Cell Culture and Human Islet Isolation and Culture

Clonal INS-1 832/13 cells expressing the human insulin gene (a gift from Dr Christopher Newgard, Duke University),¹⁴ were grown in culture at 37°C, 5% CO₂ in 75 cm² cell culture T-flasks (Corning Inc., Corning, NY). Standard RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, Mo) was supplemented with 10% fetal calf serum (Mediatech, Herndon, Va), 10 mmol/L HEPES (Media-tech), 2 mmol/L L-glutamine (Sigma-Aldrich), 1 mmol/L sodium pyruvate (Sigma-Aldrich), and 50 µmol/L 2-mercaptoethanol (Sigma-Aldrich). Cells were grown to 60% to 80% confluence. Human islets were isolated and cultured as described previously.¹

Heat Treatment of INS-1 Cells and Human Islets

For INS-1 cell heat treatment (HT) media was aspirated from each flask and cells treated with 3 mL of 0.05% trypsin/EDTA (Invitrogen, Eugene, Ore). After 2 minutes of incubation, trypsin was neutralized with 6 mL of standard serum-supplemented culture media.¹⁴ The cell suspension was then transferred to a 15-mL conical tube and placed into a 60 °C water bath for 60 minutes. For time course experiments, aliquots of INS-1 cells were removed from the 15-mL conical tube at specified timepoints (1, 3, 5, 25, and 47 hours). Heat treatment of human islets was performed by transferring 3000 islet equivalents (IEQ) in CMRL 1066 media to a conical tube, which was placed in a 60°C water bath for 5 hours. In the case of the human islet time course experiments, aliquots of 200 IEQ in CMRL 1066 media added to 1.5 mL microcentrifuge tubes were placed in a 60°C water bath for 5 hours. Aliquots were removed from the water bath and placed on ice at the specified timepoints (15 minutes, 30 minutes, and 1, 3, 5, and 30 hours). The time 0 samples were placed on ice immediately after preparation. Samples were prepared in triplicate.

Preparation of Cellular Mixtures of Known Viability

HT cells were considered 100% nonviable (dead), whereas healthy, untreated, surface-attached cells were considered 100% viable. Mixtures containing 0%, 20%, 50%, 80%, and 100% HT (dead) cells were prepared from proportioned HT and untreated INS-1 cells or human islets based on DNA measurements. DNA content in healthy and HT cells was determined using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen) per manufacturer's instructions. Samples were analyzed using a SpectraMax M5 plate reader system (Molecular Devices, Sunnyvale, Calif).

ADP and ATP Measurement With Luminescence-Based Assays

The CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp, Madison, Wisc) was used per manufacturer's instructions to measure ATP. The ApoGlow BioAssay (Adenylate

Nucleotide Ratio Assay) kit (Cambrex Bio Science Rockland, Inc., Rockland, Me) was used for ATP/ADP determination per manufacturer's instructions as described previously.⁶ Sample preparation for analysis using the CellTiter-Glo and ApoGlow assays kit was performed in the same manner: three 100- μ L cell suspension samples were taken from each mixture immediately after preparation. Each sample was diluted 10-fold in Dulbecco's phosphate buffered saline (DPBS, Mediatech, Herndon, Va) and sonicated at an amplitude of 11% for 15 seconds (Fisher Scientific, Sonic Dismemberator Model 500). Samples prepared per kit instructions were plated in 96-white-well plates (Corning 3912, Corning Inc.) for luminescence readings on a SpectraMax M5 (Molecular Devices) plate reader. Serially diluted ATP (Sigma-Aldrich) was used as a standard in both the CellTiter-Glo and ApoGlow assays. To measure DNA content, the same samples, which had been analyzed using the CellTiter-Glo and ApoGlow kits for ATP and ADP/ATP measurement were diluted an additional 10-fold in an aqueous solution of 1 mol/L ammonium hydroxide (Mallenckrodt, St. Louis, Mo) and 3.4 mmol/L Triton X-100 (Sigma-Aldrich). DNA content was then determined using the Quant-iT PicoGreen dsDNA assay kit per manufacturer's instructions.

Electrospray Tandem Mass Spectroscopy (LC/MS/MS) Analysis of Nucleotides

LC/MS/MS was used to measure the concentrations of purine nucleotides in cell samples.¹⁵ Tandem mass spectroscopy provides an advantage over high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection in that each purine nucleotide is monitored by unique ion pairs, thereby minimizing artifactual signals from co-eluting metabolites. Viability mixtures and time course samples were centrifuged at 175 $\times g$ for 4 minutes at 4°C and the supernatant was aspirated. Cells were resuspended in a 50:50 mixture of water and acetonitrile containing 2 mmol/L ammonium acetate and subsequently sonicated at an amplitude of 11% for 15 seconds with a Sonic Dismemberator (Model 500, Fisher Scientific). Samples were stored at -80°C until shipped to Yale University on dry ice for assay.

ATP and ADP concentrations in samples of INS-1 and islet cell extracts were measured with an API 4000 Qtrap LC/MS/MS (PE Sciex, Foster City, Calif) equipped with a turbo ion spray as described previously.^{15,16} D4-Taurine was used as an internal standard for quantification of MS/MS ion intensities.

RESULTS

A typical set of ATP and ADP measurements obtained for untreated, healthy INS-1 cells using the ApoGlow BioAssay kit following manufacturer's instructions as well as published protocols⁶ is presented in Figure 1. Based on the published protocol, the ADP/ATP ratio is calculated by dividing the difference in relative light units (RLU) between points *C* and *B* as indicated in the curve (a measure of the amount of ADP in the sample) by the RLU measured at point *A* (a measure of the amount of ATP in the sample). According to the protocol $ADP/ATP = (C - B)/A$. The protocol expectation is that the value for *C* will be higher than *B*, resulting in a positive *C - B* value and thus positive ADP value and ADP/ATP ratio. Although ATP measurements yielded positive and reasonable values (see point *A* on Fig 1), negative ADP values were observed (point *C* in experimental data was lower than point *B*). ADP values obtained from INS-1 cell and human islet samples and ADP standards with this method were negative in the vast majority of the cases. Human islets as well as mixtures of ATP and ADP standards sent to a collaborating lab at the University of Pennsylvania examined the samples with the same ApoGlow kit yielded negative ADP values, and thus ADP/ATP ratios, confirming our observations. The negative values obtained for ADP do not have a physical meaning, were deemed unreliable, and limited the utility of the ADP/ATP ratio generated by this method.

Consequently, LC/MS/MS was utilized as an alternative method for the measurement of high-energy phosphates. It was used to successfully measure ADP and ATP, as well as to calculate ADP/ATP ratios in INS-1 cells and human islets of varying viability. ATP and ADP measurements on HT INS-1 cells and human islets conducted with LC/MS/MS analysis confirmed that intracellular ATP as well as ADP content was effectively zero within 30 minutes of HT in a 60°C water bath. On the contrary, the DNA content in the same samples as determined using the Quant-iT Pi-coGreen DNA assay remained constant, even 2 days after initiation of heat treatment. These findings confirm that DNA, as measured with these assays was significantly more stable under conditions of cellular stress or death than ATP and ADP. The differences between the kinetics of degradation for ATP, ADP, and DNA in cells undergoing heat treatment are demonstrated in Figure 2. Using the LC/MS/MS measurements of ATP and ADP of 100% HT and 100% healthy INS-1 cells and human islets, we estimated the expected ATP/DNA and ADP/ATP ratios in mixtures of healthy and HT INS-1 cells and human islets. Based on the data presented in Figure 2, we assumed that ATP and ADP in dead cells was equal to zero and that the DNA in healthy and HT cells was constant (for HT cells this is true for at least 2 days as indicated in Fig 2). Equation (1) was used to estimate the ADP to ATP ratio of the mixtures,

$$\left(\frac{ADP}{ATP}\right)_{Mixture} = \frac{x \cdot ADP_{HC} + (1-x) \cdot ADP_{HTC}}{x \cdot ATP_{HC} + (1-x) \cdot ATP_{HTC}} \quad (1)$$

and equation (2) was used to estimate the ATP/DNA of the mixtures,

$$\left(\frac{ATP}{DNA}\right)_{Mixture} = \frac{x \cdot ATP_{HC} + (1-x) \cdot ATP_{HTC}}{x \cdot DNA_{HC} + (1-x) \cdot DNA_{HTC}} \quad (2)$$

where x is the fraction of healthy cells, $(1-x)$ is the fraction of HT cells in the mixtures, HC denotes healthy cells and HTC denotes heat treated cells. Using equations 1 and 2 and the assumptions listed, we generated values for ATP/DNA and ADP/ATP ratios for mixtures with varying proportions of HT and healthy INS-1 cells or islets. The values were normalized and expressed as a percentage of those measured in 100% healthy cells. These values were plotted versus the fraction of HT cells in mixtures of HT and healthy cells (Fig 3). These results illustrated that ATP/DNA correlated linearly with the viability expected in mixtures of healthy and HT (dead) cells, whereas ADP/ATP did not.

Normalized ATP/DNA ratios measured in INS-1 cell preparations with three methods (CellTiter-Glo, ApoGlow, and LC/MS/MS) exhibited linear correlations with the fraction of HT cells in the preparation (Fig 4). The slopes (mean values $\pm$ standard deviations) and R^2 of the lines shown in Figure 4 are estimated using linear regression of the data obtained with the three methods: -1.03 ± 0.03 , $R^2 = 0.997$ for CellTiter-Glo; -1.00 ± 0.06 , $R^2 = 0.956$ for ApoGlow; and -1.01 ± 0.04 , $R^2 = 0.976$ for LC/MS/MS. Similar results were obtained with human islets (data not shown). On the contrary, ADP/ATP ratios measured from the same INS-1 and human islet cell samples using either the ApoGlow bioassay or LC/MS/MS exhibited significant scatter and did not correlate with viability in mixtures of healthy and HT INS-1 cells or human islets (data not shown).

DISCUSSION

It seems rather safe to assert that intracellular DNA levels remain relatively stable during heat treatment, whereas ATP and ADP levels drop rapidly to zero (or near-zero) after application of heat stress. Both ATP and ADP levels fall so rapidly after death that any contribution of dead cells to an overall viability estimate becomes negligible. The commercially-available assays themselves may pose limitations that make this measurement practically difficult, in that they may lack adequate sensitivity to measure low ATP and ADP levels. Another issue with the ADP/ATP ratio is how this quantity is interpreted. In one study, an ADP/ATP ratio of a certain magnitude was interpreted to indicate high islet viability,⁶ whereas in a separate study, a similar ADP/ATP ratio was interpreted to suggest a cellular preparation exhibited a significant level of apoptosis.¹⁷ Additionally, a different study asserted that an elevation in cytosolic ATP, and thus a decline in the ADP/ATP ratio even below levels detected in healthy cells, was necessary for propagation of apoptosis.¹⁸ Therefore, there is even significant disagreement in the literature concerning the meaning of the ADP/ATP ratio as it relates to the quality of a cellular preparation; both high and low ADP/ATP ratios have been associated with increased cell death. It has been hypothesized that the increase in cytosolic ATP in cells undergoing apoptosis will not be sustained and that ATP as well as ADP will be depleted shortly after the execution phase of apoptosis. In this sense, the ADP/ATP ratio is strictly dependent on the timing of the measurement as it relates to the onset of apoptosis. This ambiguity in interpretation by different groups and the dependence of the ratio on the kinetics of the cell death process suggest that there is limited potential for use of the ADP/ATP ratio as a predictive tool to assess the viability of a cellular preparation before transplantation. Despite these facts, the ADP/ATP ratio, when measured properly, provides a snapshot into the bio-energetic status of a cell or tissue. It has been successfully used to assess the metabolic state of a variety of tissues under various conditions.^{19–21}

The ATP/DNA ratio suffers similar limitations, but DNA does not degrade as quickly in dead cells as either ATP or ADP. Because of this profound difference in the kinetics of degradation of DNA under conditions of stress, the ATP/DNA ratio permits the inclusion of dead cells into a viability estimate once the apoptotic process is fully executed. Consequently, the ATP/DNA ratio may provide a more accurate assessment of islet cell viability compared to with the ADP/ATP ratio. This was clearly demonstrated in the current study with HT INS-1 cells and islets. Additional studies are necessary to establish whether the mode of cell death or the type of lethal stress has an impact on the kinetics of nucleotide and DNA degradation and how that affects viability estimates based on either the ATP/DNA or the ADP/ATP ratio under actual islet manufacturing and culture conditions.

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Fig 1.

Trace of recordings for an ATP and ADP/ATP ratio measurement in an individual sample using the ApoGlow kit according to the included protocol. Data illustrates the mean of five wells from a sample of healthy, untreated INS-1 cells. The dotted line illustrates the expected kinetics of the assay. ADP/ATP ratio is calculated by dividing the difference in RLU between points C and B (a measure of the ADP content in the sample) by the RLU measured at point A (a measure of the ATP content in the sample). Accordingly $ADP/ATP = (C - B)/A$.

Fig 2.

ATP and DNA content measured in INS-1 cells and ATP and ADP content measured in human islets plotted versus the time from the onset of heat treatment (HT) in 60°C water bath.

Measurements were conducted with the method indicated in the legend for Figure 1. ATP, ADP, and DNA content are expressed as percent of time zero measurements (before the onset of HT). Data illustrates the mean of three separate experiments ($n = 3$), with triplicates taken for each sample. Note the staggered x -axis scale.

Fig 3.

Predicted ADP/ATP and ATP/DNA ratios in mixtures of healthy and HT cells. Ratios were calculated assuming zero ATP or ADP present in the HT cells. HT cells were regarded as possessing measurable levels of only DNA, which was supported by our measurements shown in Figure 2 as well as LC/MS/MS measurements from samples of HT cells. Predictions for ADP/ATP and ATP/DNA are projected to mixtures containing close to but not equal to 100% HT cells or islets. In samples containing 100% HT cells or islets the ADP/ATP that is undetermined (0/0), and the ATP/DNA ratio is zero.

Fig 4.

ATP/DNA ratio presented as a function of the fraction of dead cells in mixtures of HT and healthy INS-1 cells. ATP/DNA values for mixtures of healthy and HT INS-1 cells were normalized to the ATP/DNA value measured in healthy cells and are reported as percentages of that value. Data illustrate three methods of measuring ATP from the same original samples. Samples were measured in triplicate with each method.