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Adenosine kinase inhibition selectively promotes rodent and porcine islet β-cell replication

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Contributed by Douglas A. Melton, January 21, 2012 (sent for review October 1, 2011)

Diabetes is a pathological condition characterized by relative insulin deficiency, persistent hyperglycemia, and, consequently, diffuse micro- and macrovascular disease. One therapeutic strategy is to amplify insulin-secretion capacity by increasing the number of the insulin-producing β cells without triggering a generalized proliferative response. Here, we present the development of a small-molecule screening platform for the identification of molecules that increase β-cell replication. Using this platform, we identify a class of compounds [adenosine kinase inhibitors (ADK-Is)] that promote replication of β cells in three species (mouse, rat, and pig). Furthermore, the replication effect of ADK-Is is cell type-selective: treatment of islet cell cultures with ADK-Is increases replication of β cells but not that of α cells, PP cells, or fibroblasts. Short-term in vivo treatment with an ADK-I also increases β-cell replication but not exocrine cell or hepatocyte replication. Therefore, we propose ADK inhibition as a strategy for the treatment of diabetes.

Results

Development and Performance of a High-Throughput Primary β-Cell Replication Assay. To identify compounds that increase β-cell replication, we developed a screening platform using freshly isolated rat islet cells (Fig. 1A). Although the use of primary cells limits the supply of β cells and might be expected to introduce variability between culture preparations, this approach maximizes retention of in vivo metabolic characteristics pertinent to the mitotic behavior of β cells. Our cultures contained ∼75% β cells/β cells (pancreatic and duodenal homeobox 1; PDX-1+) $\sim$ 18% α cells (glucagon$^+$), $\sim$ 3% fibroblasts (vimentin$^+$), and $\sim$ 5% other cell types (Fig. 1B). Although the mixed cell type composition of our culture complicates analysis of β-cell replication, the presence of multiple cell populations within a single well is also advantageous as it allows one to determine whether compound treatment has a β-cell-selective effect: an important feature of a useful therapeutic.

We used the transcription factor PDX-1 to identify β cells (28). Although PDX-1 is expressed by both β and δ cells, the vast majority of PDX-1$^+$ cells are insulin$^+$ β cells (Fig. 1C). We used nuclear PDX-1 staining as our primary β-cell marker because islet cells grow in dense irregular clusters that cause a cytoplasmic stain, such as insulin, to be ambiguously associated with multiple nuclei. As a result of this ambiguity, ki-67$^+$ nuclei from rapidly replicating cells such as fibroblasts have the potential to be incorrectly attributed to insulin$^+$ cells. The basal in vitro β-cell replication rate showed moderate inter-experiment variability (0.4–3.5%) and was typically higher than the in vivo β-cell replication rate ($0.8 \pm 0.2\%$) of animals of a similar age, as determined by the percentage of PDX-1$^+$ cells that coexpressed ki-67.

Adenosine Kinase Inhibitors Promote β-Cell Replication. We screened ∼850 compounds from a carefully selected library of cell-permeable bioactive compounds for their ability to increase β-cell replication with the intent of finding compounds for the treatment of T2DM (27). Unfortunately, this study did not identify compounds with β-cell-selective replication-promoting activity. Therefore, it is likely that these molecular targets have an unacceptable risk profile for use in vivo. One explanation for why only nonspecific mitogenic compounds were found is that the screen was performed on a reversibly transformed cell line that may no longer retain metabolic characteristics of primary β cells. Here, we present the development of a screen for compounds that specifically promote primary β-cell replication. Using this platform, we have identified a class of compounds that selectively increase β-cell division and reveal a metabolic governor of β-cell proliferation.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1201149109/-/DCSupplemental.

PNAS | March 6, 2012 | vol. 109 | no. 10 | 3915–3920

www.pnas.org/cgi/doi/10.1073/pnas.1201149109
repliation, as measured by the percentage of PDX-1+ cells that coexpressed ki-67 (Fig. S1). Although replication rates were determined using automated image acquisition and analysis, dividing PDX-1+ cells (yellow arrows) are easily distinguished from dividing PDX-1− cells (white arrows) by the colocalization of PDX-1 and ki-67 with visual inspection (Fig. 2A). Two hit compounds were identified, both well-characterized adenosine kinase (ADK) inhibitors (ADK-Is). 5-Iodotubercidin (5-IT) (CAS 24386–93–4) and ABT-702 (CAS 214697–26–4) increased the percentage of dividing PDX cells two- to threefold above the background, independent of a variable baseline PDX cell replication rate, and have a significant effect on PDX cell number (see below). To confirm the results using PDX-1 to identify β cells, similar experiments were performed using PDX-1 and insulin co-staining to identify β cells (Fig. S2A). Indeed, insulin+ cell proliferation is enhanced by ADK-I treatment. To confirm that ADK is the likely target, we tested additional ADK-Is for their ability to promote β-cell replication. Two additional ADK-Is that demonstrate similar efficacies to the primary hit compounds are shown (Fig. S2 B and C) (29).

Next, we tested the replication effect of ABT-702 on murine and porcine islets to exclude the possibility that these findings are unique to rat β cells. For the porcine β-cell studies, 2- to 3-y-old pigs were selected to test whether enhanced replication would be seen in older animals. Compound treatment of islet cells from both of these species and rats caused a dose-dependent induction of PDX cell replication (Fig. 2B and Fig. S3 A and B). Although 5-IT is more potent than ABT-702, both compounds have a maximum induction of approximately two- to threefold. One notable observation is that the analysis of ABT-702 at concentrations above ∼20 μM is limited by background fluorescence. In addition, the replicative effect of these compounds was confirmed using both the mitotic phase marker phosphohistone-H3 (PH3) and bromodeoxyuridine (BrdU) incorporation (Fig. S3 C–F). The ability of the ADK-Is to cause a similar induction of β-cell replication across multiple species and induce the expression or incorporation of multiple replication markers (ki-67, PH3, BrdU) confirm that these compounds activate a conserved mitotic pathway in β cells. Furthermore, culturing islet cells in the presence of the 5-IT for 6 d significantly increased the number of PDX-1+ cells compared with DMSO-treated cultures (Fig. 2C). At day 6, the number of PDX cells in the 5-IT-treated wells had increased by 40% compared with a 20% increase compared to DMSO-treated wells and suggests a change from a basal replication rate of ∼3% per day to ∼6% per day in our cultures.

**ADK Is Expressed by β Cells and Negatively Regulates β-Cell Replication.** ADK is a member of the sugar kinase group of enzymes, composed of three families (hexokinases, ribokinases, and galactokinases) that play important roles in cellular metabolism (30). ADK is a ribokinase that regulates the intracellular and extracellular adenosine levels through its ability to catalyze the phosphorylation of adenosine to AMP using ATP as the phosphate donor (31). Although this enzyme is broadly expressed, it is highly expressed in the liver and pancreas (32). ADK has two known forms, a long
nuclear isoform and a short cytoplasmic isoform (33). Although isoform-specific functions have not been established, cytoplasmic ADK may preferentially participate in the purine salvage pathway, whereas the nuclear isoform functions as a global regulator of methyltransferase reactions via the feedback regulation of adenosine on S-adenosylhomocysteine hydrolase activity (34, 35). Inhibition of ADK has the dual effect of increasing extracellular adenosine and inhibiting methyltransferase reactions.

ADK immunostaining of islet cultures revealed nuclear expression of ADK in β cells. In contrast, ADK staining was in the cell body (primarily nuclear), adipose (mixed cytoplasmic and nuclear), and muscle (primarily cytoplasmic; we noted high and low ADK-expressing fiber bundles, which may reflect the different muscle fiber types) tissue sections (Fig. 1B). The presence of nuclear staining in β cells, but not α cells, indicates that the long form of ADK is expressed in β cells and not α cells.

We next tested whether the ADK in β cells acts as a negative regulator of replication. Lentiviral infection was used to direct the expression of GFP and either a nontargeted inhibitory RNA (RNAi) or one targeted to ADK. The ability of the ADK-directed RNAi to decrease ADK protein levels was confirmed by Western blot and immunostaining (Fig. S4 C and D). By infecting approximately half of the islet cell culture, as determined by GFP expression, we could separately analyze the PDX cell replication rate of infected and noninfected cells within the same well. If ADK acts as a cell-autonomous regulator of β-cell replication, then PDX cells that receive the negative control plasmid and PDX cells that remained uninfected would have the same replication rate, whereas PDX cells infected with the ADK-targeted siRNA virus would have an increased replication rate. Indeed, the results confirmed this prediction (Fig. 3E). Uninfected PDX cells (Fig. 3E, blue bars) and control infected β cells (Fig. 3E, Left, red bar) all had basal proliferation rate of ~2%. In contrast, PDX cells that received the ADK-directed siRNA demonstrated a 2.5-fold increase in their replication rate (Fig. 3E, Right, red bar). This suggests that ADK is a cell-autonomous negative regulator of β-cell replication and is likely to be the molecular target of the ADK-Is.

**ADK-Is Promote β-Cell Replication via mTOR Activation.** Several signaling molecules have previously been shown to be involved in the regulation of β-cell replication (36–39). To explore the mechanism of ADK-Is-dependent β-cell replication, we assessed PDX-1-mediated β-cell replication rate was measured in islet cultures simultaneously treated with 5-IT plus a replication-pathway inhibitor (Fig. 4A). Whereas the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 caused a small but significantly increase in β-cell replication, the phosphoinositide 3-kinase (PI3K) inhibitor wortmannin and the mammalian target of rapamycin (mTOR) inhibitor rapamycin both suppressed ADK-Is-dependent β-cell replication. Although the SB203580 increased β-cell replication in conjunction with 5-IT, we did not see this effect when this compound was tested alone. The inhibitory effects of wortmannin and rapamycin suggest that activity of the PI3K/mTOR signaling pathway might mediate the β-cell replication response to ADK-Is. To test this hypothesis, we treated the rat β-cell line INS-1E cells with ADK-Is and determined the phosphorylation status of the ribosomal protein S6 (RPS6), a downstream target of mTOR (40). Indeed, treatment with either 5-IT or ABT-702 resulted in increased RPS6 protein phosphorylation. Similar but less pronounced results were obtained when intact primary rat islets, which comprise multiple cell types, were treated with ABT-702 (Fig. S5A). These results suggested a molecular mechanism by which ADK-Is promote β-cell replication is likely via activation of the mTOR signaling pathway. It is interesting to note that mTOR is both a cytoplasmic and nuclear kinase, and this may be relevant to the presence of nuclear ADK within β cells (41).

**ADK-Is and Glucose or GLP-1R Agonists Have an Additive Effect on β-Cell Replication.** Hyperglycemia is considered to be a driver of β-cell proliferation despite relatively little in vitro evidence to support this widely accepted principle (42–44). We took advantage of our β-cell replication platform and demonstrated that glucose has a concentration-dependent effect on β-cell proliferation (Fig. 4C). However, the response kinetics appears to be different from that of ADK-Is. Whereas ADK-Is increase ki-67 staining by 24 h, an effect of glucose is not seen until later (48 h). A distinct mechanism of action for glucose and ADK-Is is supported by the additive effect of 5-IT at all of the tested glucose concentrations (Fig. 4C). The combination of high glucose and 5-IT caused a fivefold induction of the replication rate above the baseline rate. The results of this experiment indicate that the addition of ADK-Is to a hyperglycemic environment might significantly increase β-cell growth in the setting of diabetes. Similar to glucose, GLP-1R agonists are able to increase β-cell replication (45). In our experimental conditions, GLP-1R agonists only modestly increased PDX cell replication (~1.5-fold) compared with 5-IT (~2.5-fold); however, the addition of 5-IT to the GLP-1R agonists led to an

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**Fig. 3.** β cells express nuclear ADK, which acts as a cell-autonomous negative regulator of proliferation. (A–C) Nuclear ADK staining (red) is present in β cells (insulin; green) (A) but not α cells (glucagon; green) (B) or fibroblasts (vimentin; green) (C). (D) Somatostatin cells (green) demonstrate variable intensities of nuclear ADK expression. White arrows identify representative cells in each image. (Scale bars: 100 μm.) (E) Quantification of β-cell replication after infection with virus containing either a scrambled RNAi sequence (Left) or an ADK-directed RNAi sequence (Right). The replication rate of uninfected cells (blue) and infected cells (red) within the same well were analyzed separately on the basis of virus-encoded GFP expression. Error bars represent SEM (n = 8 independent wells); *P < 0.01. See SI Methods for additional experimental details.

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**Fig. 4.** The combination of high glucose and 5-IT caused a fivefold increase in β-cell replication rate. A) The combination of high glucose and 5-IT caused a fivefold increase in β-cell replication rate. B) The combination of high glucose and 5-IT caused a fivefold increase in β-cell replication rate. C) The combination of high glucose and 5-IT caused a fivefold increase in β-cell replication rate. D) The combination of high glucose and 5-IT caused a fivefold increase in β-cell replication rate. E) The combination of high glucose and 5-IT caused a fivefold increase in β-cell replication rate.
ADK-Is Selectively Promote β-Cell Replication. We assessed the replication rate of multiple cell types in our islet culture: pancreatic polypeptide (PP) cells, α cells, δ cells, and fibroblasts (Fig. 4A and B). We expected that δ cells might show increased replication in response to ADK-Is because, like β cells, δ cells express nuclear ADK (Fig. 3D), secrete their hormone in response to glucose, and share the expression of several key transcription factors including PDX-1 (46). Indeed, δ cells did demonstrate a significant increase in replication in response to ADK-I treatment, whereas fibroblasts, α cells, and PP cells did not (Fig. 5B). The replication rate of PP cells is not shown because their division is extraordinarily rare in culture. In addition to β cells, hepatocytes also express high levels of nuclear ADK and, therefore, might be expected to proliferate in response to ADK-Is (Fig. S4B) (35). However, hepatocyte replication is not increased in response to drug treatment (Fig. 5C). Therefore, the replication effect of ADK-Is is selective and not solely dependent upon nuclear ADK expression.

ADK-Is Promote β-Cell Replication in Vivo. Encouraged by the in vitro results, we tested whether ABT-702 could selectively promote β-cell replication in vivo. ABT-702 was chosen because of its longer half-life compared with 5-IT (47). Indeed, a single intraperitoneal (i.p.) injection of ABT-702 resulted in a twofold increase in BrdU incorporation by PDX-1 cells (Fig. 5D and Fig. S5D). This result was confirmed in two separate cohorts of animals in which (i) β cells were identified by the presence of insulin rather than PDX-1 and (ii) replicating cells were identified by the presence of ki-67 rather than BrdU (Fig. S6). Notably, treatment with ABT-702 did not increase the replication rate of exocrine cells, again highlighting the selectivity of ADK-Is (Fig. 5F and Fig. S6F). In addition, BrdU incorporation by hepatocytes was examined in response to ABT-702 treatment (Fig. 5G), and these cells did not show an increased rate of cell division. Therefore, ABT-702 selectively promotes β-cell replication in vitro and in vivo.

Discussion

Historically, T2DM therapies have attempted to augment insulin secretion (e.g., sulfonylureas) or reduce insulin demand (e.g., biguanides, thiazolidinediones) by lowering peripheral resistance. However, T2DM patients appear to have a limited capacity for adaptive β-cell expansion, and these approaches do not address this deficiency. Here, we present a platform to identify pharmacological agents that promote increased β-cell division. In contrast to previously identified growth-promoting compounds, which lack cell type specificity (27, 48–49), we find that ADK-Is, which lack cell type specificity, as a class of agents that is capable of promoting β-cell replication in vitro and in vivo. Of critical importance is that these compounds preliminarily appear to have a selective proliferation effect on β cells and not α cells, hepatocytes, exocrine cells, or fibroblasts. The molecular mechanism for β-cell selectivity is not apparent. Although we believe that the presence of nuclear ADK may be relevant to the observed selectivity, other cell types, such as hepatocytes, also have nuclear ADK but do not replicate in response to ADK-Is. We hypothesize that intracellular (possibly nuclear) adenosine levels within β cells activate the mTOR pathway with unique consequences within β cells. A notable limitation of the current study is that we have tested the proliferative effect of ADK-Is on a restricted number of cell types. This issue and whether ADK inhibition can promote the replication of normal and diabetic human β cells will need to be addressed in future studies.

The primary physiological cue for β-cell replication is thought to be glucose (44). Because the diabetic condition is defined by insufficient insulin supply despite excess glucose, diabetes, in part, represents a failure of the compensatory β-cell response to excess glucose. Here, we have identified a pharmacological stimulus for β-cell replication that is glucose-independent and, therefore, has the potential to circumvent defects in the glucose-response pathway. The additive replication effect of ADK-Is and GLP-1R agonists raises the possibility for simultaneous use of these agents in the treatment of T2DM. An important question
is whether long-term in vivo treatment with ADK-Is will improve glucose tolerance by augmenting β-cell mass in rodents and humans. To answer this experimental question, it may be necessary to develop ADK-Is with improved potency (the identified compounds are active in the 1–10 μM range) and less central nervous system accessibility to avoid central nervous system effects that have been observed with ADK inhibition (50).

Although inhibition of ADK causes cellular efflux of adenosine, we do not believe that the growth-promoting effect of ADK-Is on PDX-1⁺ cells is mediated by paracrine/autocrine adenosine signaling (51). First, we observe a cell-autonomous increase in β-cell proliferation in response to ADK knockdown. If this effect were mediated by extracellular adenosine, a paracrine effect would be anticipated. Second, the addition of adenosine receptor agonists and antagonists that were included in our screening libraries had no effect on β-cell proliferation in our assay. Third, β-cell replication in response to ADK-Is requires mTOR activity. Interestingly, prior work has suggested that mTOR activity is directly influenced by cellular ATP levels and that mTOR can be found in the nucleus of normal cells (41, 52). Whether the nuclear localization of ADK in β cells and the primary function of ADK in maintaining nucleotide pools is relevant to the mechanism of mTOR activation and the induction of β-cell replication by ADK-Is remains to be determined.

The identification of ADK as a regulator of β-cell replication is an unexpected finding that highlights the value of using chemical screening to reveal new biology. The methodology established here using primary islet β cells may be usefully applied to study the identification of compounds that promote the regeneration of other tissues such as cardiac myocytes and neurons, which demonstrate limited postnatal cellular division.

**Methods**

**Islet Isolation and Primary Screen Protocol.** Rat (250-g Sprague–Dawley; Charles River) and mouse (12-wk-old animals; C57BL/6; Jackson Laboratory) islets were isolated as described previously (53). The use of animals was approved and carried out in accordance with our institutional animal care and use committee. Porcine islets from retired breeders were provided by VitaCyte. Islets were incubated (37 °C; 5% CO₂) overnight in islet media [99–786-CV (Mediatech); 10% (vol/vol) FBS serum (Valley Biomedical); BS3033; 8.3 mM glucose (Sigma; G7528); 1× penicillin/streptomycin (Invitrogen; 15070–063); 1× Glutamax (Invitrogen; 35050–079)]. The following morning, islets were trypsinized into cellular clusters of 1–3 cells, resuspended in islet media and plated into the wells of a 96-well plate (Sigma; CLS3904) that had been coated with 804G (a rat bladder carcinoma cell line) conditioned media. The cellular plating density was 70,000 cells/well, and >95% viability was confirmed at the time of plating. The islet cells were allowed 48 h to adhere; at that time, the media were changed (as above, except 2% (vol/vol) serum, 5 mM glucose), and the cells were compound-treated. For screening, compounds were tested at 1 and 10 μM concentrations in duplicate on a single occasion. After 24 h of compound treatment, cells were fixed with fresh 4% (wt/vol) paraformaldehyde. See SI Methods for additional details regarding immunohistochemical and automated replication analysis.

**Quantification of in vivo Replication.** Twelve-week-old C57/B6 female animals were injected with BrdU (Sigma; B5002; 10 μl/kg) and with either ABT-702 (21 mg/kg) or DMSO vehicle. Twenty-four hours posttreatment, the animals were killed, and the relevant organs were harvested. A similar experiment was performed in 6-wk-old C57/B6 female animals without BrdU treatment. In these animals, replication was assessed after 24 h by ki-67 staining. All experiments were performed with a minimum of four animals per treatment condition.

Fig. 5. ADK-Is selectively promote β-cell replication in vitro and in vivo. (A) Representative images used to analyze the replication rates of PP cells (pancreatic peptide), α cells (glucagon), δ cells (somatostatin), and fibroblasts (vimentin) present in the in vitro rat islet culture. The molecular marker of cell identity is red, ki-67 is green, and nuclear staining is blue. The red box within the glucagon-stained image (Top Right) is a high magnification view of two ki-67 and glucagon double-positive cells (arrow). (B) Quantification of the in vitro replication rates of δ cells (somatostatin); Top Left, α cells (glucagon); Top Right, and fibroblasts (vimentin); Bottom Left) after treatment with DMSO, 5-IT (2 μM), or ABT-702 (15 μM). *P < 0.01 and **P < 0.05 compared with DMSO-treated condition. (C) Quantification of the replication rate of isolated murine hepatocytes grown in the presence of EGF (40 ng/ml) and HGF (20 ng/ml) plus DMSO or ABT-702 (15 μM). See SI Methods for additional experimental details. (D) Representative image used to quantify in vivo replication in animals treated with either DMSO or ABT-702. Sections were stained for PDX-1 (green), BrdU (red), and DNA (blue). (E–G) Quantification of the in vivo replication rates of islet β cells (E), exocrine cells (F), and hepatocytes (G). Error bars represent the SD, P values were obtained using two-tailed t test.
group. Every fourth 12-μm section was used for analysis, and for a minimum of 2,000 β cells, exocrine cells, and hepatocytes per organ per animal were counted. Analysis was performed by manual picture acquisition and cell counting. β Cells were identified by either PDX-1 staining or insulin staining, with similar results. Exocrine cells were approximated by counting all nuclei outside the islet structure. A minority of these cells were not exocrine cells. Hepatocytes were identified as DAPI+ albumin+ cells (Bethyl Laboratories; A90-234A). Dividing cells were BrdU+ (Amersham; RPN202).

**Statistics.** Data are presented as the means of multiple replicates performed simultaneously. All of the experimental results presented were repeated more than twice. Error bars show the SD unless otherwise specified. Results were compared using the two-tailed t test. The EC50 was calculated using nonlinear regression with the highest replication rate constrained.

**ACKNOWLEDGMENTS.** We thank Yuval Dor, Qiao Zhao, Deni Cohen, Jayaraj Rajagopal, Julie Sneddon, and Susan Bonner-Weir for their thoughtful input throughout the development of this work. We are grateful for the technical support of George Kenty, Kathryn Koszka, Bridget Molloy, Josh Cohen, Vaja Tchpashvili, and Karolina Siniakowicz. This work was supported by the Howard Hughes Medical Institute (D.A.M.), National Institutes of Health Grants DK084206 (to J.P.A.) and U01DK072505 (to D.A.M.), and the Diabetes Research and Wellness Foundation (G.W.).