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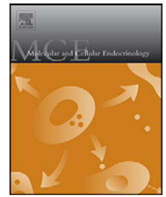

AGI-1067, a novel antioxidant and anti-inflammatory agent, enhances insulin release and protects mouse islets

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ABSTRACT

The antioxidant and anti-inflammatory compound AGI-1067 (succinobucol) has potential as an oral anti-diabetic agent. AGI-1067 reduces H_bA1c, improves fasting plasma glucose, and reduces new-onset diabetes. We investigated AGI-1067 for possible effects on mouse pancreatic islets *in vitro*. Pretreatment with 10 μM AGI-1067 increased glucose-stimulated insulin secretion (11 mM) without affecting secretion in basal (3 mM) glucose. AGI-1067 enhanced the intracellular calcium response to glucose stimulation in 7 mM and 11 mM glucose, but had no effect in 28 mM or basal glucose. AGI-1067-pretreated islets also showed enhanced calcium responses to methyl pyruvate and alpha-ketoisocaproate at low doses, but not high doses. The AGI-1067-mediated effects on glucose-stimulated calcium were maintained during continuous diazoxide exposure, suggesting effects on the K_{ATP}-channel-independent pathway. AGI-1067 also reduced cytokine-induced islet cell death and expression of iNOS, a key component in cytokine signaling. This is the first report of direct stimulatory and protective effects of a first-in-class potential anti-diabetic agent on pancreatic islets.

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1. Introduction

Type 2 diabetes mellitus (T2D) is a disease characterized by insulin resistance and the progressive failure of pancreatic beta cells to secrete sufficient amounts of insulin to regulate blood glucose. The increased incidence of T2D world-wide has led the CDC to classify it as an epidemic. Insufficient levels of insulin can lead to hyperglycemia, which promotes the progressive deterioration of insulin-producing beta cells and leads to more severe and chronic health problems including high blood pressure, neuropathy, vision loss, renal failure, stroke, coma, and death. Although the pathophysiological mechanisms involved in the development of T2D are complex, oxidative stress and inflammation are known key contributors to the development of the disease (Evans et al., 2002; Hotamisligil, 2006). Prolonged hyperglycemia can lead to oxidative stress and activation of resultant inflammatory pathways, which is important not only in insulin resistance (Shoelson et al., 2006), but also pancreatic beta-cell failure (Robertson et al., 2004; Kaneto et al., 2005; Oprescu et al., 2007).

Several antioxidants, including the anti-hyperlipidemic drug probucol, have been shown to preserve beta-cell mass and function, improve glucose tolerance, and prevent the development of diabetes in several preclinical models of diabetes by reducing the oxidative stress that promotes beta-cell apoptosis (Drash et al., 1988; Uehara et al., 1991; Fukuda et al., 1995; Gorogawa et al., 2002; Takatori et al., 2003; Lankin et al., 2004).

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The clinical use of probucol, however, has been associated with side effects, including reduced high-density lipoprotein cholesterol (HDLc) and prolonged QT interval, thus limiting its usage (Tardif et al., 1997). AGI-1067 [mono[4-[[1-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-1-methylethyl]thio]-2,6-bis(1,1-dimethylethyl)phenyl]ester] (butanedioic acid), also called succinobucol, is a metabolically stable derivative of probucol (Meng et al., 2002, 2004) that is currently in clinical development for the treatment of diabetes. Clinical studies have shown that AGI-1067 inhibits both restenosis (Tardif et al., 2003) and atherosclerosis (Tardif et al., 2008a) and does not show QTc prolongation (Tardif et al., 2003). More recently, a double-blind, placebo-controlled trial in subjects with established cardiovascular disease demonstrated that AGI-1067 significantly reduced the composite of cardiovascular death, cardiovascular arrest, myocardial infarction or stroke (Tardif et al., 2008b). Furthermore, in the diabetic subpopulation of this study, AGI-1067 significantly reduced glycated hemoglobin levels and improved fasting plasma glucose levels. In addition, AGI-1067 substantially reduced the incidence of new-onset diabetes. The magnitude of the preventative effect was greater than that seen with the anti-diabetic drugs acarbose and metformin and comparable to thiazolidinediones. Unlike thiazolidinediones however, AGI-1067 did not cause increased weight or waist circumference (Tardif et al., 2008b).

Preclinical mechanistic studies have shown that AGI-1067 is not only a potent lipid antioxidant, but also an anti-inflammatory agent. Specifically, AGI-1067 inhibits inflammatory signaling pathways and reduces the expression of inflammatory adhesion molecules and cytokines in several cell types including endothelial cells and macrophages (Kunsch et al., 2004; Luyendyk et al., 2007). In addition to those properties, AGI-1067 has also been shown to enhance insulin sensitivity in mouse adipocytes by blocking inflammatory signaling pathways known to contribute to peripheral insulin resistance (Chen et al., 2008). Furthermore, in animal studies, AGI-1067 inhibited the progression of atherosclerosis (Sundell et al., 2003) and improves insulin resistance (Sundell et al., 2008).

To date, AGI-1067 has not been studied at the level of the pancreatic islet. Given the potential importance of oxidative stress and inflammatory processes on islet cell dysfunction in diabetes, we investigated AGI-1067 for effects on islets *in vitro* using a newly developed fluorescent imaging technique. By fluorescently labeling one set of islets with Cell Tracker Red (CTR) for positive identification, we could distinguish between unlabeled and CTR-labeled islets to record both control and AGI-1067-treated islets simultaneously and under identical experimental conditions to monitor changes in intracellular $[Ca^{2+}]_i$ as a measure of normal islet function (Henquin et al., 2006; Jahanshahi et al., 2009). This technique improved our sensitivity in evaluating this potential therapeutic compound for effects on islet function. Our results indicate that pretreatment with AGI-1067 protects islets against cytokine-induced damage and also increased calcium influx and insulin secretion in response to glucose stimulation among control islets. Our study suggests AGI-1067 has multiple direct effects on islet function and demonstrates the utility of a dual-labeling technique for testing potential therapeutic compounds on islet function.

2. Materials and methods

2.1. Mice and islet isolation

Male C57BL/6J mice weighing 20–35 g were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a pathogen-free facility at the University of Virginia (UVA) for use in all studies. Mice were euthanized according to IACUC approved protocol, and their pancreatic islets were isolated by collagenase digestion and Histopaque centrifugation as previously published (Carter et al., 2009). Following isolation, islets were transferred to a Petri dish containing RPMI 1640 (Invitrogen Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin/streptomycin. All islets were incubated overnight to allow sufficient recovery time from collagenase digestion before any experiments were performed.

2.2. Drug treatments

AGI-1067 was supplied by AtheroGenics, Inc. and solubilized in DMSO (0.1% DMSO for final concentration). Cell Tracker Red (CTR, a cell permeable mildly thiol-reactive vital probe used to label cells) and fura-2 AM (a probe for $[Ca^{2+}]_i$) were purchased from Invitrogen. Murine cytokines (B&D Scientific, Mountain Lakes, NJ) were solubilized in sterile H_2O at 1000× for use at the following final concentrations: 100 pg/ml for TNF-alpha, 1000 pg/ml for IFN-gamma, and 50 pg/ml for IL-1beta in KRB. This combination of cytokines is within the range of concentrations we have previously published (Yang et al., 2005; Jahanshahi et al., 2009). Unless otherwise stated, all other drugs used were purchased from Sigma-Aldrich (St. Louis, MO) and made soluble in 0.1% DMSO.

2.3. Cell Tracker Red labeling and $[Ca^{2+}]_i$ measurements of islet function

$[Ca^{2+}]_i$ was measured using the ratiometric $[Ca^{2+}]_i$ indicator fura-2 AM using previously described methods (Jahanshahi et al., 2009). Islets were dye-loaded and recorded in a modified Krebs-Ringer buffer (Nunemaker et al., 2004) containing (in mM): 11 glucose, 130.5 NaCl, 3 $CaCl_2$, 5 KCl, 2 $MgCl_2$, 10 HEPES, pH 7.3 (3, 7, or 28 mM glucose was used in place of 11 mM as indicated below). Islets were loaded for 30–40 min with 1 μM fura-2 AM or with 1 μM fura-2 AM + 0.2 μM CTR, washed, and then transferred to a small volume chamber (Warner Instruments, Hamden, CT) mounted on the stage of an Olympus BX51WI fluorescence microscope (Olympus, Tokyo, Japan). Islets were recorded in 3 mM (low) glucose for 3 min and then exposed to 7, 11, or 28 mM (high) glucose stimulation, or other treatments as described. Every experiment was performed with at least two trials using islets isolated from different mice on separate occasions and with CTR labels flipped to further verify CTR labeling did not interfere with islet calcium handling. The glucose-stimulation $[Ca^{2+}]_i$ response (GSCa) is defined as the change in $[Ca^{2+}]_i$ levels between high vs. low glucose as measured by fura-2 AM ratio (340/380 nm fluorescence). Data were analyzed with IP Lab software Version 4.0 (Scanalytics, Rockville, MD).

2.4. Cell death measurements

Measurements of cell death were performed by treating islets with 20 μg/ml of propidium iodide (PI) for 10 min. Islets were imaged once under brightfield illumination to determine the islet borders and imaged again to measure PI fluorescence using 535 nm excitation and 617 nm emission. AnnexinV (Invitrogen), which detects cells that have expressed phosphatidylserine on the cell surface, was also used (488 nm excitation, 535 nm emission).

2.5. Islet insulin secretion

After overnight incubation, islets were tested for insulin secretion as described previously (Chen et al., 2002; Nunemaker et al., 2008). Briefly, islets were preincubated at 37 °C and 5% CO_2 for 1 h in a modified Krebs–Ringer Buffer (KRB) solution containing 0 mM glucose, then washed and incubated in KRB supplemented with 3 mM glucose for 1 h followed by a 1-h treatment with KRB containing 11 mM glucose. The supernatant was collected after each treatment, and insulin concentration in the supernatant was measured by an EIA method (Mercodia, Uppsala, Sweden) with a mouse insulin standard. The intra-assay variation was 3.6% and inter-assay variation was <10%.

2.6. RT-PCR

RNA from islets was prepared using the RNeasy kit (Qiagen, Valencia, CA). cDNA was made from 5 μg of total RNA using MMLV reverse transcriptase in 20 μL reaction volume using random hexamers (Invitrogen). For quantitative measurement of PCR products, 3 μL of the cDNA reaction (5-fold diluted) was used as template for PCR with Jump Start Taq-Polymerase (Sigma-Aldrich, St. Louis, MO) in a reaction volume of 25 μL for PCR (Chakrabarti et al., 2002). Taqman probes were purchased from Applied Biosystems (Applied Biosystems, Carlsbad, CA) and real-time PCR was performed according to manufacturer's instructions. All thermal cycling was performed using the CFX96 Thermal Cycler (Bio-Rad, Hercules, CA). All reactions were performed in triplicate and the data was normalized to the housekeeping gene actin and evaluated using the $2^{-\Delta\Delta CT}$ method. Expression levels are presented as fold induction/downregulation of transcripts of respective genes relative to control.

2.7. Statistics

A two-tailed Student's *t*-test was used for comparisons of control vs. AGI-treated conditions and for cytokine vs. cytokine + AGI-1067 conditions. For gene expression studies, we used one-way ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered significant.

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