Use of a Soluble Tetrazolium Compound to Assay Metabolic Activation of Intact β Cells

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Although assessments of metabolic activation are central to studies of β-cell function, available techniques are tedious, insensitive, and/or require cell disruption. We have investigated the use of a new water-soluble tetrazolium salt, MTS (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H -tetrazolium, inner salt), in the presence of phenazine methosulfate (PMS), an intermediate electron acceptor that amplifies its signal (fluorescence at 490 nm). During static incubations of glucose-responsive (HIT-T15 or INS-1) dispersed β cells with increasing glucose concentrations, there was a progressive increase in MTS reduction, with a maximum signal-to-noise (S/N) ratio of 24 with HIT-T15 cells and 10 with INS-1 cells. This was associated with, but not attributable to, parallel increases in insulin secretion. Pure mitochondrial fuels (α-ketoisocaproate [KIC], methyl pyruvate [MP], or L-glutamine [GLN] + L-leucine [LEU]) also increased the reduction of MTS in INS-1 cells (6.5-, 4.8-, and 14.4-fold, respectively), but generally less than glucose, suggesting a major role of glycolysis in the signal induced by glucose. Inhibitors of glucose metabolism (mannoheptulose [MH], Iodoacetate [IA], or 2-deoxyglucose [2-DG]) markedly reduced the glucose-stimulated MTS signal. In comparison to another tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), MTS assay provided a better S/N ratio with glucose or other nutrient secretagogues. Extant theory holds that activation of mitochondrial dehydrogenases by increments in Ca^{2+} influx couples glycolysis to mitochondrial oxidation of glucose-derived fuels. However, reduction of fuel-induced calcium influx (by Ca^{2+}-free medium or diazoxide [DZX]) or direct stimulation of calcium influx (by 40 mmol/L K^+) failed to significantly modulate the signal, arguing against this theory. We conclude that the MTS assay is a facile test that reflects the global metabolic function of insulin-secreting β cells. Furthermore, since this assay does not require disruption of cells to solubilize the formazan product, and therefore also allows concomitant measurement of insulin secretion, it offers considerable advantages over earlier methods.

ALTHOUGH ASSESSMENTS of metabolic activation are central to studies of β-cell function, available techniques are tedious, insensitive, involve the use of radiolabeled compounds, and/or require cell disruption. Colorimetric tests based on the reduction of tetrazolium salts have been used to study metabolic activity of many cell types, including intact islets and β cells.[1] These methods rely on the concept that tetrazolium salts are reduced to their respective formazan compounds by metabolically active cells. Although originally it was believed that mitochondrial dehydrogenases mediated the effect, more recent studies principally implicate extramitochondrial NADH and NADPH.[2,3] The use of one tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), necessitates the use of a solubilization protocol to dissolve MTT-formazan crystals formed intracellularly.[4] A wide range of solvents has been used for this purpose (acidified isopropanol, dimethyl formamide/sodium dodecyl sulfate [SDS], SDS, dimethyl sulfoxide [DMSO], etc), some of which require removal of medium from cells before elution of crystals.[3] More importantly, these solvents disrupt the cells' integrity, necessitating that measurements of insulin secretion be performed either before or in parallel (but not concurrently) with the MTT assay.

We have investigated the use of a new water-soluble tetrazolium salt, MTS (3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H -tetrazolium, inner salt), in the presence of phenazine methosulfate (PMS), an intermediate electron acceptor that transfers electrons to MTS and amplifies its signal, in assessing the metabolic activity of insulin-secreting (HIT-T15 and INS-1) β cells. One of the advantages of the MTS test is that it readily exits intact cells, obviating the need to disrupt the cells and thereby permitting concurrent measurements of insulin release. We therefore standardized and evaluated the MTS assay using β cells, and then used it to examine further an extant theory that activation of mitochondrial dehydrogenases by an increment in mitochondrial Ca^{2+} concentrations (itself promoted by increments in Ca^{2+} influx into the β cells) couples glycolysis to mitochondrial oxidation of glucose-derived fuels.[5,6] Since this theory has recently been disputed,[7] we reexamined it using the MTS test.

MATERIALS AND METHODS

Insulin-Secreting Cells and Materials

HIT-T15 (passages 70 to 78) were provided by Dr Hui-Jan Zhang and Dr Paul Robertson (University of Minnesota Medical School, Minneapolis, MN) and INS-1 cells (passages 65 to 76) were provided by Dr Claes Wollheim (University of Geneva, Geneva, Switzerland). Details of culture conditions and the insulin secretory capacity of HIT and INS-1 cell lines in response to various secretagogues have been described previously.[8-11]

MTS and PMS (Celltiter 96 aqueous nonradioactive cell-proliferation assay) were obtained from Promega (Madison, WI); L-glutamine (GLN) was obtained from Gibco Laboratories (Long Island, NY); and α-ketoisocaproate (KIC), methyl pyruvate (MP), L-leucine (LEU), mannoheptulose (MH), iodoacetate (IA), 2-deoxyglucose (2-DG), antimycin (AM), diazoxide (DZX), 3-*O*-methyl glucose (3-MG), clonidine (CLN), somatostatin (SS), and MTT were purchased from Sigma Chemical (St Louis, MO).

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MTS Assay

The MTS assay essentially followed directions provided by Promega. HIT-T15 or INS-1 cells were cultured in 24-well plates. Each well contained approximately 2×10^6 cells at the time of testing. The cells were preincubated and incubated in Krebs-Ringer bicarbonate (KRB) buffer, which was supplemented with HEPES (10 mmol/L) and gassed with 95% O_2 and 5% CO_2. Grade V bovine serum albumin (Sigma) or delipidated albumin (ICN Immunobiologicals, Costa Mesa, CA) 0.1% were used as described in the Results. MTS and PMS were added to KRB to achieve final concentrations of 0.04 mg/mL and 0.03 mmol/L, respectively, in the incubation medium. Unless specified, the cell cultures were washed with an MTS/PMS-free and glucose-free KRB buffer, preincubated at 37°C for 30 minutes in the same buffer, and then incubated at 37°C for 30 minutes with MTS/PMS-containing KRB with or without glucose and other test substances as specified. At the end of the incubation period, the medium bathing the cells was aspirated, centrifuged at 6,000 rpm for 6 minutes, and the supernatant used to measure optical density and insulin secretion. Optical density was recorded with a microplate reader (model EL 312; Biotek Instruments, Winooski, VT) at 490 nm, using a 96-well plate. Readings were corrected for background optical density by subtracting the readings from MTS/PMS-containing KRB incubated in the same water bath at 37°C for 30 minutes in the absence of β cells.

Initially, we studied the effect of the combination of MTS/PMS present during the preincubation period alone. With this protocol, there was no discernable MTS signal, indicating that MTS/PMS freely exited the cells and was therefore aspirated immediately before the incubation period. Therefore, MTS and PMS were always added to the incubation medium. However, there was no significant difference in MTS signal when MTS and PMS were present during both preincubation and incubation periods as compared with their presence only during incubation alone (data not shown). Therefore, for all subsequent studies, MTS/PMS was present only for the incubation period. Moreover, when we studied the effect of nutrients on MTS alone (in the absence of PMS), there was no discernable signal, implying the crucial role of PMS in the transfer of electrons from reducing equivalents to MTS.

Insulin Release

Concurrent insulin release into the incubation medium was also measured. Immunoreactive insulin was measured by radioimmunoassay (RIA) using rat insulin as standard and a guinea pig antipork insulin antibody and expressed as microunits per well.

MTT Assay

The MTT assay was performed as described previously.[1,4] MTT was dissolved in KRB buffer (5 mg/mL) and filtered using a syringe filter (0.2 μm) to remove insoluble particles. At the end of a 30-minute preincubation period with MTT-free KRB buffer in a 24-well plate (similar to that for the MTS assay), MTT (0.5 mg/mL)-containing buffer (along with the test substances) was added and incubated at 37°C for for an additional 30 minutes. To dissolve formazan crystals at the end of incubation period, the medium was aspirated out, 375 μL of isopropanol was added to each well, and the plate was agitated in a plate shaker at room temperature for 5 to 10 minutes. We tested an alternative solubilization protocol using DMSO (375 μL for 30 minutes), and did not find it superior to isopropanol. Therefore, we used isopropanol for all our MTT studies. Optical density was then measured at 562 nm as described earlier. Previous investigators had noted increases in baseline "noise" when optical density was assessed in the presence of albumin or medium, especially that containing bicarbonate.[3,12] Therefore, to reduce the high background MTT signal (obtained in the absence of glucose or nutrient secretagogue), we included an additional step of washing the wells with 0.9% sodium chloride solution, after aspirating out the medium at the conclusion of incubation. We also used delipidated albumin instead of bovine serum albumin (BSA) for the MTT assay to minimize any contribution to background MTT reduction attributable to the metabolism of exogenous fatty acids. Insulin secretion was measured in the medium obtained at the end of incubation.

Data Expression and Statistical Analyses

Except in analyses of signal-to-noise (S/N) ratio (ie, the ratio between the signal obtained with maximal glucose concentration used and that obtained in the absence of glucose), all stimulatory responses (MTS signal or insulin secretion) were expressed as the incremental response. For the MTS test, they were computed as absolute signal (in absorbance units [AU]) in the presence of agonist subtracted by the signal in its absence. Inhibitory responses were calculated using the ratio of control responses (denominator) and the signal in the presence of the inhibitor (numerator). Data are expressed as the mean ± SE throughout. Except where indicated, "n" refers to the number of experiments, where each experiment generally contained three to six determinations per condition.

Statistical analyses were performed using the Student's *t* test or the Mann-Whitney rank-sum test as appropriate, using a *P* value less than .05 as statistically significant.

RESULTS

Responses to Fuels

There was a progressive increase in MTS reduction with increasing glucose concentration (Fig 1). These responses were nearly linear over time (Fig 2). With HIT-T15 cells, a maximum S/N ratio of 24.1 ± 2.4 (mean ± SE; n = 4 experiments) was obtained. A ratio of 10 ± 1.2 (n = 3 experiments) was obtained with INS-1 cells. Half-maximal responses were obtained with 1.3 mmol/L glucose in HIT-T15 cells and 2.4 mmol/L in INS-1 cells. These responses were associated, pari passu, with increases in insulin secretion in both cases (Fig 1). On the contrary, 3-MG (10 mmol/L), a nonmetabolizable analog of glucose, failed to stimulate either MTS reduction or insulin secretion in HIT-T15 cells (not shown). The basal MTS signal varied between studies from 0.01 to 0.05 AU, contributing in a major degree to the variability in the S/N ratio; in contrast, in the presence of an agonist (eg, 11.1 mmol/L glucose), the variability within each experiment was small (coefficients of variation, 9.6% and 3.4%, respectively; n = 12 replicates using HIT cells).

In INS-1 cells, the pure mitochondrial fuel, KIC (20 mmol/L), provided in the absence of glucose, increased the absorbance ($P < .0001$; n = 4 experiments) and concurrent insulin secretion ($P < .0001$) (Fig 3A). Similarly, MP (20 mmol/L), the methyl ester of pyruvate which is preferentially metabolized in the mitochondrial domain of islets,[13] increased the reduction of MTS ($P < .001$; n = 3 experiments) and insulin secretion ($P = .002$) in INS-1 cells (Fig 3B). A combination of mitochondrial fuels, GLN (2 mmol/L) and LEU (20 mmol/L) also increased the MTS signal (control, 0.01 ± 0.00 AU; GLN + LEU, 0.05 ± 0.00; $P < .001$; n = 2 experiments) and insulin secretion (control, 369 ± 26 μU/well; GLN + LEU, 635 ± 39; $P = .0001$) in INS-1 cells. However, note that these increments in MTS signal were considerably less than those induced by glucose ($P < .001$ to .04). Similarly, in HIT-T15 cells, 20 mmol/L KIC induced a significant, but small increase in MTS reduction (no-glucose control, 0.04 ± 0.00 AU; KIC,

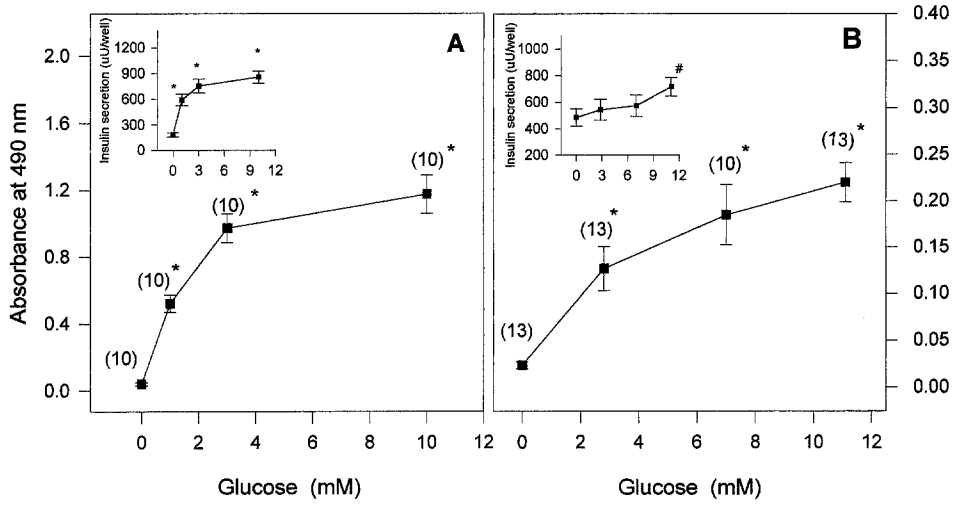

Fig 1. Glucose dose-response relationship (A, HIT-T15 cells; B, INS-1 cells). Main panels show MTS reduction and insets show insulin secretion in response to glucose. Cells were incubated for 30 minutes. Data are means ± SE. Numbers in parentheses indicate number of individual determinations. *$P < .001$, #$P = .02$ *v* corresponding value obtained in the absence of glucose.

0.14 ± 0.00, compared with glucose 10 mmol/L, 1.10 ± 0.02; n = 2 experiments; $P = .002$ between basal and KIC); and insulin secretion (control, 256 ± 20 μU/well; KIC, 443 ± 34; high-glucose control, 1,666 ± 148; $P = .0009$ between control and KIC). Together, these studies suggest that the MTS assay may reflect cytosolic (glycolytic) events more sensitively than mitochondrial metabolism.

Responses to Secretory or Metabolic Inhibitors

To exclude the possibility that MTS is taken into insulin secretory granules and discharged into the medium during exocytosis, the effects of CLN (1 μmol/L) or SS (1 μmol/L) were tested. In HIT-T15 cells, neither CLN nor SS inhibited glucose (10 mmol/L)-induced MTS reduction; however, both agents significantly inhibited insulin secretion (Fig 4). These findings suggest that MTS and PMS do not passively exit the β cells along with insulin during exocytosis.

The effects of inhibitors of glucose metabolism were then studied. MH (30 mmol/L), a specific inhibitor of high-capacity hexokinase (glucokinase; GK), reduced the glucose (11.1 mmol/L)-stimulated MTS signal in INS-1 cells (n = 3 experiments; $P < .001$) (Table 1). Concurrently, insulin secretion was reduced from 607 ± 45 μU/well to 356 ± 23 μU/well ($P < .001$). These findings are compatible with significant expression of GK in INS-1 cells.[14] On the contrary, in HIT-T15 cells, in which glucose phosphorylation is thought to be predominantly a function of the low-K_m hexokinase (HK),[15] MH (30 mmol/L) did not significantly reduce the MTS signal

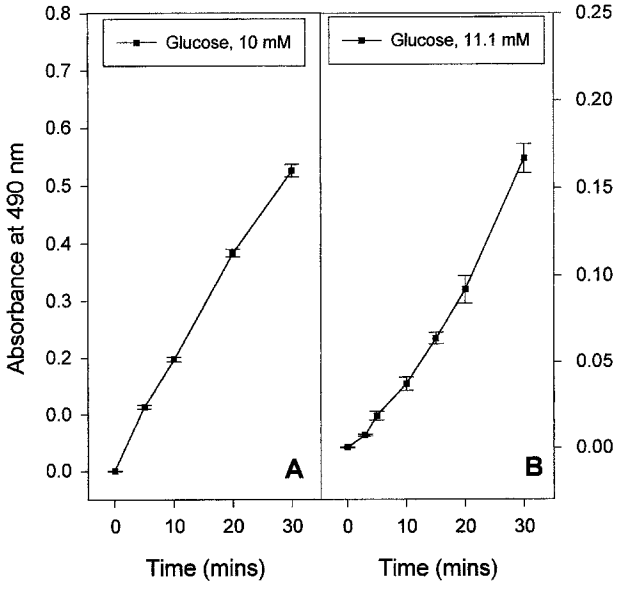

Fig 2. DMTS assay in dispersed (A, HIT-T15 cells; B, INS-1 cells) β cells showing time-course responses to high glucose. Data (means ± SE) are representative of 2 similar experiments each. Absolute absorbance units were computed after subtracting the respective (ie, timed) cell-free background signal.

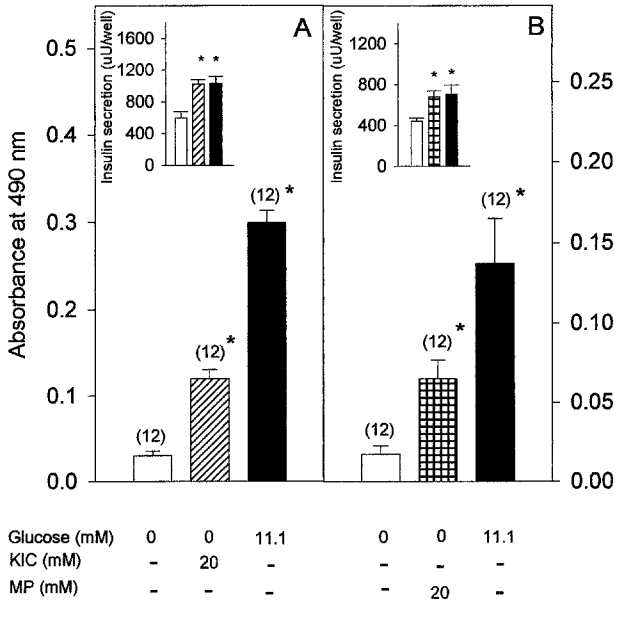

Fig 3. Effects of pure mitochondrial fuels on MTS reduction (main panel) and insulin secretion (inset) in INS-1 cells: (A) effects of KIC, and (B) effects of MP. Cells were incubated for 30 minutes. Data are means ± SE. Numbers in parentheses indicate number of individual determinations. *$P < .0001$ *v* corresponding value obtained in the absence of glucose or test substance.

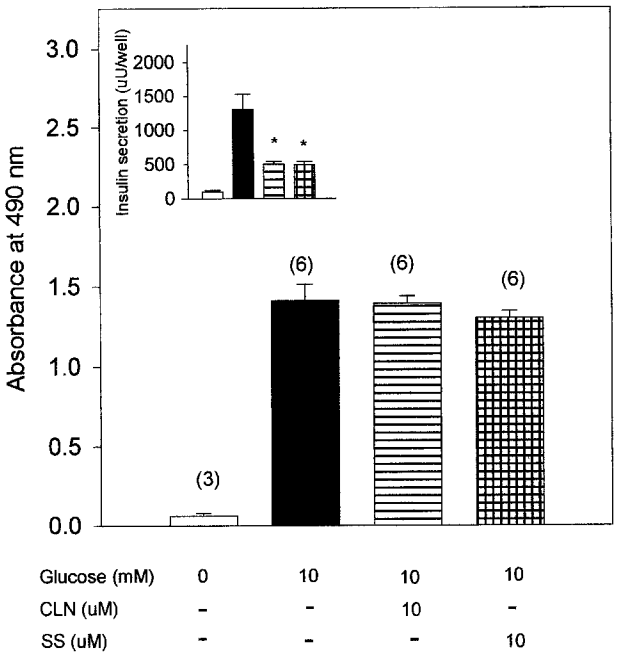

Fig 4. Effects of inhibitors of insulin secretion (CLN and SS) on glucose (10 mmol/L)-induced MTS reduction (main panel) and insulin secretion (inset) in HIT-T15 cells. Cells were incubated for 30 minutes. Data are means ± SE. *$P = .002$ *v* corresponding value obtained in the presence of glucose (10 mmol/L) as agonist in the absence of inhibitors. Numbers in parentheses indicate number of individual determinations.

(Table 1) or insulin secretion (glucose 0 mmol/L, 200 ± 25 μU/well; high glucose, 877 ± 90; MH, 854 ± 121; P = not significant [NS]). Conversely, 2-DG (25 mmol/L), a competitive inhibitor of HK, significantly inhibited the glucose (11.1 mmol/L)-stimulated MTS reduction in HIT-T15 cells (Table 1). In INS-1 cells, 2-DG (25 mmol/L) also blunted the glucose (11.1 mmol/L)-stimulated MTS signal (Table 1) and insulin secretion (high glucose control, 589 ± 37 μU/well; 2-DG, 407 ± 35; $P = .0012$), supporting the likely coexistence of low-K_m HK activity in addition to GK activity in INS-1 cells.[16] In HIT-T15 cells, IA (0.8 mmol/L), a glycolytic inhibitor, reduced the glucose (11.1 mmol/L)-stimulated MTS signal (Table 1) and eliminated insulin secretion (data not shown).

Since mitochondrial activity contributes to the generation of reducing equivalents, we studied the effect of AM, an inhibitor of the mitochondrial respiratory chain. In INS-1 cells, AM (10 μmol/L) significantly inhibited the glucose (11.1 mmol/L)-induced MTS reduction (Table 1) and insulin secretion (data not shown). Similarly, in HIT-T15 cells, 10 μmol/L AM reduced the glucose (10 mmol/L)-stimulated MTS signal (glucose 0 mmol/L, 0.03 ± 0.00 AU; high glucose, 0.39 ± 0.01; AM, 0.18 ± 0.01; n = 2 experiments; $P < .001$) and eliminated insulin secretion (data not shown). However, in INS-1 cells, AM (10 μmol/L) did not inhibit KIC (20 mmol/L)-stimulated MTS reduction (n = 2 experiments; data not shown), but still significantly inhibited the concurrent insulin secretion ($P = .024$) (see Discussion).

Responses to Perturbations of Ca^{2+} Fluxes

Since an increase in intramitochondrial calcium concentration might activate mitochondrial dehydrogenases,[5,6] we studied the effects of perturbations of calcium on glucose-induced MTS reduction. Reduction of fuel-induced Ca^{2+} influx in INS-1 cells (through the use of Ca^{2+}-free medium containing 500 μmol/L EGTA) failed to reduce the MTS signal measured at 30 minutes, although the concurrent insulin secretion was inhibited by 78% validating that a state of extracellular Ca^{2+} deprivation had been achieved (Fig 5A). Similarly, DZX (250 μmol/L), which opens K^+-adenosine triphosphate (ATP) channels and inhibits subsequent Ca^{2+} influx, failed to inhibit the glucose (11.1 mmol/L)-stimulated MTS signal (control, 0.21 ± 0.03 AU; DZX, 0.19 ± 0.02; n = 3 experiments; P = NS), but decreased concurrent insulin secretion significantly (control, 1,081 ± 235 μU/well; DZX, 501 ± 101; $P = .037$). Moreover, addition of 40 mmol/L KCl to the incubation medium (in the absence of glucose) did not significantly modulate the MTS signal, although insulin secretion increased significantly, as expected (Fig 5B). Similarly, 40 mmol/L KCl failed to increase the glucose (2.2 mmol/L)-induced MTS reduction in INS-1 cells (data not shown). To determine the effects of calcium at an earlier time point, we performed an MTS assay in INS-1 cells for 10 minutes; however, there still was no significant modulation of the glucose (16.7 mmol/L)-stimulated MTS signal with extracellular calcium depletion (high glucose control, 0.021 ± 0.003 AU; high glucose-Ca^{2+}_e. 0.029 ± 0.008; n = 3 experiments; P = NS). Similarly, addition of 40 mmol/L KCl to glucose-free medium failed to modulate the MTS signal (data not shown). When studied in the presence of 2.2 mmol/L

Table 1. MTS Assay in Dispersed β Cells: Effect of Inhibitors of Glucose Metabolism

HIT-T15 Cells					INS-1 Cells				
Inhibitor	LG (AU)	G10 (AU)	G10 + Inhibitor (AU)	P*	Inhibitor	LG (AU)	G11.1 (AU)	G11.1 + Inhibitor (AU)	P*
IA	0.27 ± 0.03*	0.87 ± 0.05	0.39 ± 0.02	<.0001	AM	0.01 ± 0.00†	0.13 ± 0.02	0.05 ± 0.01	.009
(0.8 mmol/L)	(7)	(12)	(12)		(10 μmol/L)	(12)	(12)	(12)	
2-DG	0.20 ± 0.01*	1.12 ± 0.13	0.44 ± 0.07	<.001	2-DG	0.02 ± 0.00†	0.17 ± 0.02	0.09 ± 0.01	.0015
(25 mmol/L)	(7)	(7)	(7)		(25 mmol/L)	(13)	(13)	(13)	
MH	0.05 ± 0.01†	1.12 ± 0.06	1.02 ± 0.03	.233	MH	0.02 ± 0.01†	0.18 ± 0.02	0.08 ± 0.01	<.001
(30 mmol/L)	(9)	(9)	(9)		(30 mmol/L)	(10)	(10)	(10)	

NOTE. Data are means ± SE for the number of separate determinations given in parentheses. Each experiment consisted of a preincubation step (30 minutes) in the absence of glucose or test substance followed by incubation (30 minutes) in the presence of the test substance with/without glucose.

Abbreviations: AU, absorbance units measured at 490 nm; LG, low glucose (*0.5 mmol/L glucose, †0 mmol/L glucose); G10, 10 mmol/L glucose; G11.1, 11.1 mmol/L glucose.

**P* value between high glucose alone *v* high glucose plus inhibitor.

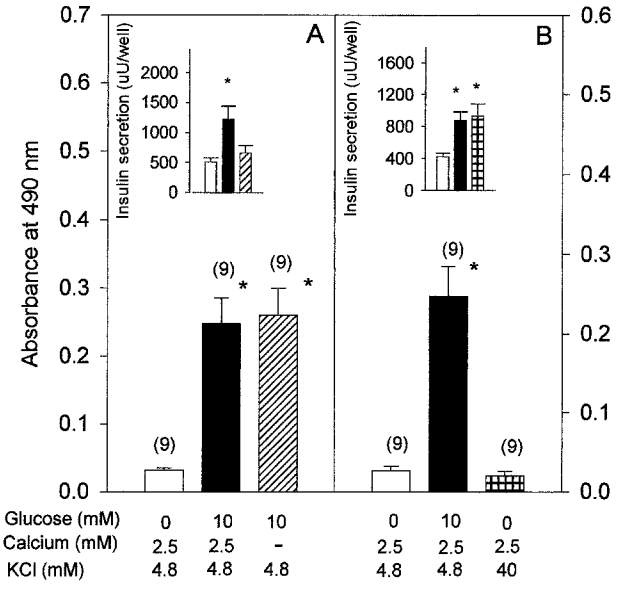

Fig 5. Effects of perturbations of calcium influx on MTS reduction (main panel) and insulin secretion (inset) in INS-1 cells: (A) effects of extracellular Ca^{2+} depletion by use of Ca^{2+}-free medium in the presence of 500 μmol/L EGTA ($-Ca^{2+}_e$), and (B) effects of 40 mmol/L K^+ in normal Ca^{2+}-replete incubation medium. Cells were incubated for 30 minutes. Data are means ± SE. Numbers in parentheses indicate number of individual determinations. *$P < .01$ *v* corresponding value obtained in the absence of glucose studied in the presence of 2.5 mmol/L Ca^{2+} in the incubation medium.

glucose in the incubation medium, addition of 40 mmol/L KCl did result in a significant, but small increase in the MTS signal (control, 0.007 ± 0.002; high K^+, 0.016 ± 0.002; n = 3 experiments; $P = .006$).

Effect of MTS/PMS on Insulin Secretion

The combination of MTS/PMS did not inhibit basal or glucose-stimulated insulin secretion in either cell line. On the contrary, MTS/PMS increased the basal and glucose (11.1 mmol/L)-stimulated insulin secretion in INS-1 cells by 37% ($P = .035$) and 42% ($P = .046$), respectively (n = 3 experiments). Similarly, in HIT cells, there was a 36% and 27% increase in basal and glucose (10 mmol/L)-stimulated insulin secretion, respectively ($P = .001$ and $P = .012$, respectively; n = 2 experiments). These compounds did not affect the insulin RIA directly.

Comparison to MTT Assay

Using the MTT assay, glucose (11.1 mmol/L) increased the signal from 0.76 ± 0.03 to 1.0 ± 0.05 AU, 0.30 ± 0.01 to 0.49 ± 0.01 AU, and 0.40 ± 0.01 to 0.46 ± 0.02 AU in three separate experiments. Thus, the S/N ratio (mean, 1.4) obtained was considerably less than that seen using the MTS assay, which was mainly attributable to much higher and more variable basal optical density measurements, ie, 0.28 to 0.81 AU in the MTT assay as compared with 0.01 to 0.05 AU in the MTS assay. Likewise, the combination of GLN (2 mmol/L) plus LEU (20 mmol/L) only increased the MTT signal by 21% (control, 0.24 ± 0.03 AU; GLN + LEU, 0.29 ± 0.04; n = 2 experiments; P = NS).

DISCUSSION

Since insulin secretion from β cells is intimately linked with the uptake and metabolism of glucose and other fuels, assessments of the metabolic activity of insulin-producing cells are of paramount importance. During the process of fuel metabolism, reducing equivalents are generated in cytosolic and mitochondrial compartments of β cells,[17,18] yielding a quantifiable signal. During static incubations of dispersed β (HIT-T15 or INS-1) cells, glucose increased the signal derived via the reduction of MTS in a time- and dose-dependent fashion, associated with parallel increases in insulin secretion. Pure mitochondrial fuels (KIC, MP, or GLN + LEU) also increased the MTS signal significantly; however, these increases were substantially less than those induced by glucose, suggesting the central importance of extramitochondrial, glycolytically derived reducing equivalents (NAD[P]H) for generation of the MTS signal. Indeed, a prevailing concept is that transfer of glycolytically derived reducing equivalents into mitochondria via discrete (glycerol phosphate and malate-aspartate) shuttles is coupled to increments of the (ATP)/(adenosine diphosphate [ADP] + P_i) ratio and subsequent insulin secretion.[19,20]

Appropriate inhibitors of glucose metabolism markedly reduced the glucose-stimulated MTS signal. MH (30 mmol/L), an inhibitor of GK, reduced the glucose-stimulated MTS signal in INS-1 cells, but not in HIT cells. However, IA (0.8 mmol/L), an inhibitor of glyceraldehyde phosphate dehydrogenase, and 2 DG (25 mmol/L), an inhibitor of HK, inhibited the glucose-induced MTS reduction in HIT-T15 cells. These findings agree with the concept that metabolism of glucose is predominantly glycolytic in HIT-T15 cells, but that GK, which is present in only low amounts in HIT cells, provides only a minor amount of glucose-6-phosphate, whereas the low-K_m HK is of greater quantitative importance, in contrast to INS-1 cells.[15,16,21] This conclusion is supported by the modest shift to the right in the glucose dose–metabolic response relationship in INS-1 cells (Fig 1). This curve is further shifted to the right in intact pancreatic islets, for which the metabolic "pacemaker" is largely GK.

AM, an inhibitor of the mitochondrial electron transport chain, inhibited the glucose-stimulated MTS signal. However, AM failed to significantly block the effect of 20 mmol/L KIC on MTS reduction in INS-1 cells, although it was effective in reducing the secretory response to KIC. Taken together, these findings are compatible with an indirect, extramitochondrial effect of AM in these cells, that an inhibition of the electron transport chain leads to accumulation of reducing equivalents, which would then inhibit glycolysis, probably at the level of glyceraldehyde-3-phosphate dehydrogenase, after reaching the cytosol via mitochondrial shuttles. This formulation is compatible with the findings in several studies, which demonstrated that the use of respiratory chain inhibitors like azide, cyanide, or AM was associated with an accumulation of cellular-reduced pyridine nucleotides.[16,22-24] The weaker effect of KIC on MTS reduction, compared with glucose, supports the formulation that the MTS signal predominantly reflects extramitochondrial reduced pyridine nucleotides, and supports the conclusions of Marshall et al[3] and Berridge and Tan[2] that the role of mitochondrial dehydrogenases in the reduction of tetrazolium salts has been greatly overstated in the past.[25,26]

A prevalent theory holds that intramitochondrial dehydrogenases are activated by increments in intramitochondrial Ca^{2+} secondary to nutrient-induced increases in Ca^{2+} influx.[5,6] However some recent data challenge the physiologic relevance of Ca^{2+} effects in mitochondria. For example, the increase in intracellular Ca^{2+} concentration follows, rather than precedes, intramitochondrial oxidation of glucose-derived fuels[7] and therefore is likely a consequence and not a cause of the increase in the (ATP)/(ADP) ratio induced by nutrients. Also, in mouse β cells, glucose-induced NAD(P)H autofluorescence was unaltered by Ca^{2+} deprivation.[27] In the current studies, depletion of extracellular Ca^{2+} failed to inhibit the glucose-stimulated MTS signal. Similarly, blockade of Ca^{2+} influx using DZX did not significantly inhibit the MTS signal, and Ca^{2+} influx induced by 40 mmol/L K^+ failed to increase the MTS reduction. Since the maximal change in intramitochondrial Ca^{2+} is transient,[28] it is conceivable that a short-lived increase in the MTS signal was overlooked. At a shorter time point (10 minutes) in INS-1 cells, we observed a small, albeit significant increase in the glucose (2.2 mmol/L)-stimulated MTS reduction in the presence of 40 mmol/L KCl in the incubation medium, but no effect of 40 mmol/L KCl in the absence of glucose. These findings are reminiscent of those of Duchen et al,[27] who used mouse islet cells. These data might relate to the findings of Kennedy et al,[28] who showed that intramitochondrial calcium increases to the micromolar range (4 to 6 μmol/L) on exposure of INS-1 cells to high potassium; however, this concentration is much greater than the level resulting from maximal glucose stimulation (600 to 800 nmol/L). Together, the data suggest that the increase in intramitochondrial Ca^{2+} induced by nutrients may not be of sufficient magnitude to activate mitochondrial dehydrogenases to a physiologically significant degree, at least in these cells. Indeed, Gilon and Henquin[23] found only a minimal blunting by Ca^{2+} deprivation of the glucose-induced increase in NAD(P)H fluorescence in mouse islets.

The MTS test may be of lesser utility for free-floating, intact islets. In preliminary experiments on intact rat islets, we observed that high glucose induced only a modest incremental MTS signal. On using batches of 50, 100, or 200 islets for static incubation (60 minutes), there appeared to be a modest (33% to 73%) increment in the MTS signal, seen only when 100 or 200 islets were used per tube. Insulin secretion, measured concurrently, increased twofold to threefold (data not shown). It is possible that MTS, which is water-soluble, has limited access to the interior of islets, which contains the majority of β cells. Although using dispersed islets might improve the MTS signal, we did not pursue this further, as this would defeat the advantage of the MTS assay as a facile test. If the MTS assay is to be used on intact islets, use of immobilized islets might improve our preliminary results.

Even though the MTS assay seems to be more sensitive in reflecting glycolytic rather than mitochondrial events in β cells, it does detect mitochondrial oxidation, as shown by the response to pure mitochondrial fuels. Therefore, this assay can be used to measure the global metabolic activity of intact β cells. On the other hand, the MTT assay, as shown in the data of Janjic and Wollheim,[1] is more likely to measure intramitochondrial events, or those in the endoplasmic reticulum,[2] and is capable of detecting a quantitatable, albeit small, signal in intact islets.[1] However, the MTS assay provides a better incremental (and S/N ratio) response when studying dispersed cells. An advantage to either tetrazolium reduction test is that they do not involve the use of radiolabeled compounds, but, with the MTS assay, there is no need to disrupt cells to dissolve the formazan compound at the end of incubation. Furthermore, concurrent insulin secretion can be measured from the same medium at the end of incubation. These represent significant advantages over the MTT test. Assays of cellular-reduced pyridine nucleotides via measurement of their autofluorescence,[29,30] or via the MTT test,[1] have generally yielded only a twofold to threefold change with glucose, compared with 10- to 20-fold increments with the MTS assay. All of these features make MTS a facile, yet informative test, in studying the global metabolic activity of pure β cells.

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