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Rapid communications

Islet cell metabolism is reflected by the MTT (tetrazolium) colorimetric assay

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Summary. Insulin secretion depends critically on glucose metabolism. We investigated whether a rapid viability test could be established for assessing glucose metabolism in insulin secreting cells. The MTT (C,N-diphenyl-N′-4,5-dimethyl thiazol 2 yl tetrazolium bromide) colorimetric assay (reduction of tetrazolium salt to formazan) was applied to rat islets and rat insulinoma cell lines. It was found that the rate of formazan production correlated with glucose oxidation and glucose utilization at glucose concentrations which also stimulated insulin secretion. In differentiated insulinoma INS-1 cells, salt reduction paralleled the insulin release at glucose concentrations of up to 8.3 mmol/l. The glucose-induced formazan production in INS-1 cells and islets was abolished by exposure to the Beta-cell cytotoxic agents, streptozotocin or alloxan. The MTT assay thus provides a convenient tool for the rapid assessment of Beta-cell metabolism and viability.

Key words: Tetrazolium salt, glucose metabolism, insulin secretion, insulin-secreting cell lines, islets of Langerhans.

In experimental diabetes mellitus, it is of importance to dispose of rapid and specific tools for assessing the functional integrity of islet cells. Cell viability is generally estimated by dye exclusion tests, vital dye staining and ^{51}Cr release assays. Determination of insulin content, insulin release, insulin biosynthesis and monitoring of glucose metabolism with radioactive tracers have all been used to more specifically probe Beta-cell function. None of the latter functional evaluations is suitable for rapid and large-scale screening of, for instance, islets from higher mammals isolated for transplantation purposes.

The MTT (C,N-diphenyl-N′-4,5-dimethyl thiazol 2 yl tetrazolium bromide) colorimetric assay has been suggested to be an indirect estimation of mitochondrial oxidative processes of living cells. The test measures the reduction of the tetrazolium salt into insoluble coloured formazan crystals [1, 2]. Considering the unique metabolic feature of the test, it appears to be particularly suitable for assessing the function of the Beta cell which is capable of modulating its glycolytic and oxidative rates as a function of extracellular glucose concentrations [3]. We have characterized the MTT assay in isolated islets and in two Beta-cell lines displaying different glucose sensitivity, i.e. RIN m5F cells and INS-1 cells, a recently established insulinoma cell line with partially preserved glucose-induced insulin release at physiological glucose concentrations [4]. In addition the MTT test was also used to reveal the effects of Beta cytotoxic agents on insulin-secreting cells.

Materials and methods

Cells

RINm5F and INS-1 cells both originate from a transplantable rat insulinoma [5]. RINm5F cells (population doubling time = 24 h) were cultured as previously described [6] in RPMI 1640 (glucose 11.2 mmol/l) supplemented with L-glutamine 2 mmol/l, 10% fetal calf serum, antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml). INS-1 cells (population doubling time = 100 h) were cultured in the same medium with the following additions: Na-pyruvate 1 mmol/l, HEPES 10 mmol/l and 2-mercaptoethanol (2-ME) 50 µmol/l, complete medium [4].

Rat islets were isolated from adult male Wistar rats (200–250 g) by collagenase digestion after ductal distension and purification on a gradient made by a single layer of ficoll-histopaque 1077 (Sigma Chemicals, St Louis, Mo., USA). Hand-picked islets were kept free-floating in complete medium for 1 day prior to the experiment.

MTT assay

INS-1 cells ($3–5 \times 10^4$ cells/200 µl/well) were cultured on 96-well microplates coated with poly-L-ornithine (Sigma Chemicals) for 3–5 days. Cells were pre-incubated and incubated in a Krebs-Ringer

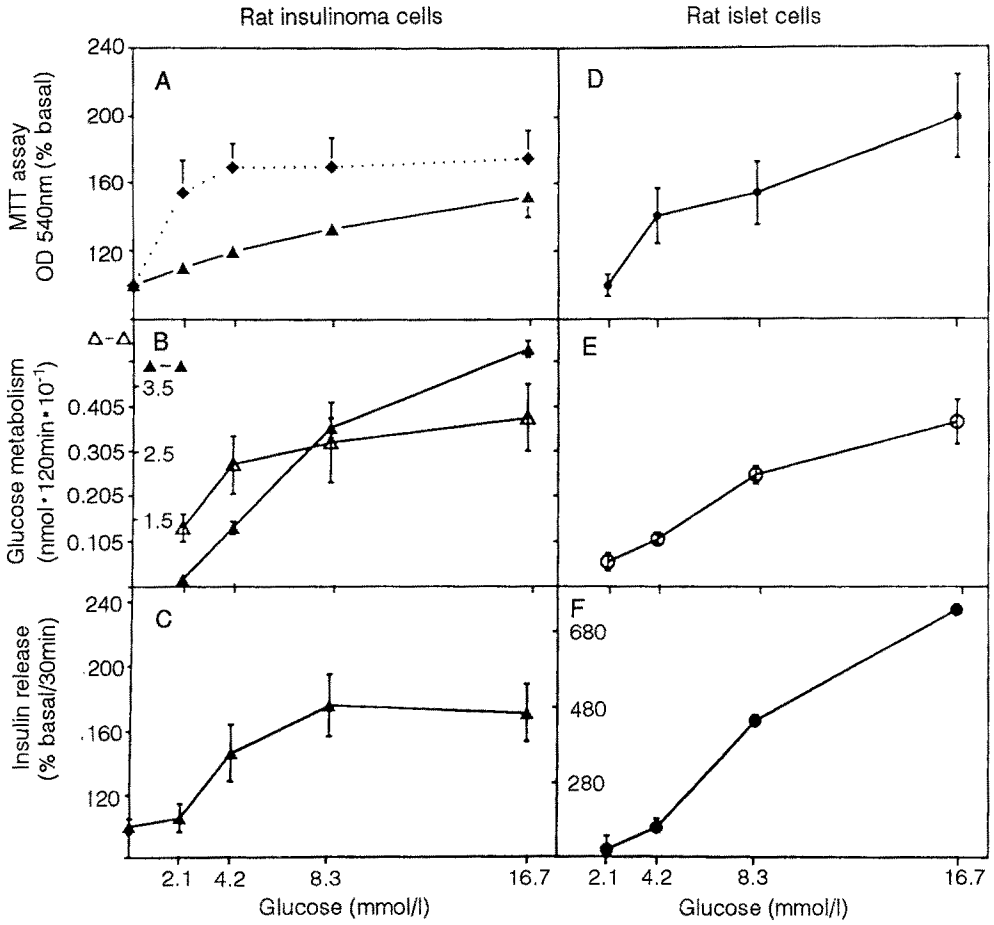

Fig. 1 A–F. Effect of glucose on MTT reduction, glucose metabolism and insulin release. Panels **A B C** illustrate studies with insulinoma cells INS-1 cells (solid lines, triangles) and RIN m5F cells (dotted line, diamonds). Panels **D E F** illustrate studies with rat islets (solid lines, circles). For the MTT assays (panels **A** and **D**), the basal values represent the formazan production (optical density) measured after 120-min incubation at 0 mmol/l glucose (**A**) or 2.1 mmol/l glucose (**D**). Glucose metabolism was estimated in INS-1 cells by [^{14}C]-U-glucose oxidation (panel **B**, open triangles) and [^{3}H]-5 glucose utilization (panel **B**, filled triangles). The values are expressed as the rate of glucose metabolism per 10^5 cells. In islets, the metabolism was estimated by the [^{14}C]-6-glucose oxidation and the values are the rate of metabolism per 40 islets. Except for panel **B** where the values are the mean ± SEM of two separate experiments (five-six replicas), all the results are the mean ± SEM of three separate experiments, each with three-five replicas. Basal insulin release (glucose 2.1 mmol/l) for islets was 0.81 ± 0.3 ng · 40 islets^{-1} · 30 min^{-1}. Basal insulin release (glucose 0 mmol/l) for INS-1 cells was: 4.2 ± 0.1 ng · well^{-1} · 60 min^{-1}

bicarbonate buffer supplemented with HEPES 10 mmol/l and bovine serum albumin 0.1% (KRB). The assay was performed as described [1]. Briefly, MTT (C,N-diphenyl-N'-4,5-dimethyl thiazol 2 yl tetrazolium bromide) (Sigma Chemicals) was dissolved in KRB (5 mg/ml), and filtered (0.45 μm) to remove insoluble particles. Unless specified the cell cultures were washed with an MTT-free and glucose-free KRB, pre-incubated at 37 °C for 60 min in the same buffer, and then incubated at 37 °C for a 120-min period with MTT (0.5 mg/ml) and different glucose concentrations. This incubation time was selected since formazan production had reached a plateau. To solubilize the formazan crystals at the end of the incubation, the medium was either removed and the cells exposed to 75 μl isopropanol or was supplemented (50% volume/volume, v/v) and incubated (3–12 h, at 37 °C) with an extraction solution (10% N,N dimethyl formamide + 40% sodium dodecyl sulphate). The optical density was recorded with an automatic spectrometer at 540 nm (Dynatech, Embrach, Embraport, Switzerland). Isolated rat islets were pre-incubated 30-min in KRB containing glucose 2.1 mmol/l, then distributed in a 96-well microtitreplate (40 islets · well^{-1} · 100 μl KRB^{-1}). After 30-min incubation a sample was taken for insulin release determination and the islets were incubated for an additional 90 min after the addition of MTT (0.5 mg/ml).

Streptozotocin and alloxan were dissolved in Na-citrate 0.2 mmol/l pH 4.5 as concentrated solutions immediatly prior to use.

Insulin release

Insulin release was measured either in parallel conditions (INS-1 cells) or prior to the exposure of the cells to MTT (islets). Immunoreactive insulin was measured by radioimmunoassay using rat insulin as a standard and a guinea-pig anti-pork insulin antibody [6].

Glucose metabolism

Glucose utilization was assessed in INS-1 cells by the conversion rate of ([^{3}H]-5) D-glucose to tritiated H_2O as already described for RIN m5F cells [6]. Glucose oxidation was measured by assessing the $^{14}CO_2$ production from either [^{14}C]-U-D-glucose (INS-1 cells) [^{14}C]-6-D-glucose (islets) after 120-min incubation at 37 °C in an inner well suspended in a scintillation vial. The $^{14}CO_2$ was trapped by the addition into the vial of 500 μl hyamine hydroxide (Sigma Chemicals) essentially as described by others [7].

Statistical analysis

Statistical analysis was performed by using the Student's *t*-test for unpaired data.

Results and Discussion

For both INS-1 cells and islets incubated in the presence of MTT, the formazan production increased as a function of the extracellular glucose concentration (Fig. 1 A and D). Experiments aiming to compare INS-1 cells with RINm5F cells were also performed (Fig. 1 A). The latter cells present abnormalities, both at the level of glucose transport and glucose phosphorylation, which prevent an increase of glycolytic flux upon an increase in the ambient glucose level at physiological glucose concentrations [6, 7]. It has

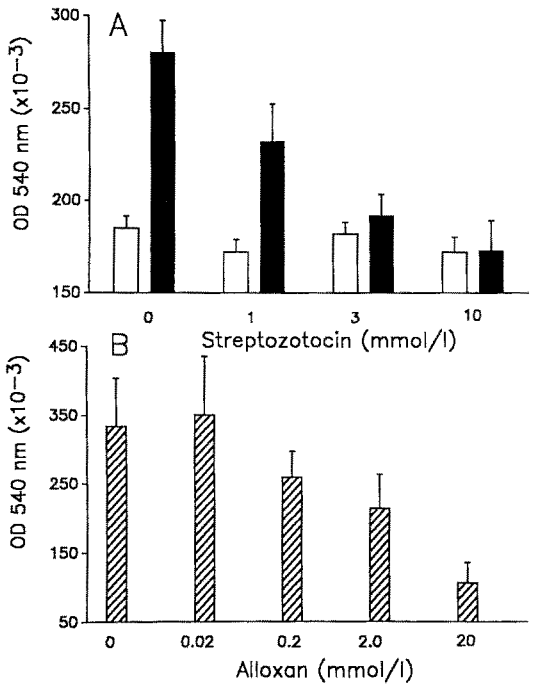

Fig. 2 A, B. Effect of streptozotocin or alloxan on MTT reduction in INS-1 cells. Streptozotocin (panel **A**) was present during the pre-incubation (60 min, glucose-free KRB), then the cells were washed and further incubated with MTT at 2.8 mmol/l (open bars) or 16.7 mmol/l (filled bars) glucose. In panel **B**, cells were pre-incubated without the drug and alloxan was added during the incubation period at 8.3 mmol/l glucose. Results are given as absolute values for optical density (OD) 540 nm. Values are mean ± SD (five replicas) of one representative experiment out of three

been shown previously that glucose utilization is not stimulated when ambient glucose is increased above 4 mmol/l [6]. This is confirmed by the results in Figure 1 A which demonstrated that in RINm5F cells no further increase in formazan production was observed at higher glucose concentrations in marked contrast to the findings in INS-1 cells or isolated islets. It should be noted that at a maximal glucose concentration (33.4 mmol/l), similar rates of formazan production were achieved in all three cell preparations (not shown).

To determine the validity of the MTT assay as an estimate of glucose metabolism, the test was performed in parallel to glucose oxidation measurements in INS-1 cells and islets, as well as to glucose utilization in INS-1 cells. The results in Figure 1 B and E indicate that glucose oxidation was increased by extracellular glucose in a dose-dependent manner from 2.1 mmol/l to 16.7 mmol/l. However, in INS-1 cells the oxidative rate did not increase further between 8.3 mmol/l and 16.7 mmol/l glucose in contrast to the situation in islets. It should be noted that when glucose utilization was measured in INS-1 cells (Figure 1 B, filled triangles) there was a 6.6-fold increase compared to the 2.8-fold increase in glucose oxidation. The oxidation / utilization ratio was lower at 16.7 mmol/l glucose (9%) than at 2.1 mmol/l glucose (22%) which suggests that, like the RINm5F cells, and in contrast to native Beta cells, INS-1 cells do not preferentially increase their oxidative metabolism at high ambient glucose concentrations [7].

That the glucose effect was dependent on the rate of metabolism was confirmed in experiments performed at 24°C. As expected, under these conditions, glucose did not increase the formazan production (not shown). In addition, MTT reduction was also markedly stimulated in INS-1 cells by the combination of L-glutamine 2 mmol/l and L-leucine 20 mmol/l (gln-leu) which is known to increase insulin release in islet cells through an increase in metabolic flux [3]. The data of one representative experiment were as follows: Formazan production: optical density 540 nm $\times 10^3$; glucose 2.1 mmol/l = 122.6 ± 4.6, glucose 2.1 mmol/l + gln-leu = 213.2 ± 1, glucose 16.7 mmol/l = 195.0 ± 11.8, glucose 16.7 mmol/l + gln-leu = 221 ± 4.1 (mean ± SD, n = 5). Thus, there was no additional effect of glucose over that exerted by glutamine and leucine in increasing formazan production.

Increasing glucose concentrations stimulated insulin release from both INS-1 cells and islets (Fig. 1 C and F). However, in INS-1 cells maximal rates of secretion were observed at 8.3 mmol/l glucose (1.8-fold increase, $p < 0.02$) compared with islets which displayed the highest rates at 16.7 mmol/l glucose (7-fold increase). Under these conditions, in contrast to insulin release, the formazan production was comparable for both types of cells over the glucose concentration range. The dissociation between the two parameters is possibly due to factors regulating insulin secretion other than metabolism which may be altered in these transformed cells.

To probe whether the MTT assay can be used for monitoring Beta-cell cytotoxicity, INS-1 cells and islets were exposed to streptozotocin or alloxan. Whereas alloxan is thought to mediate its action mainly through the generation of free radicals, streptozotocin causes direct DNA damage and a lowering of NAD and ATP levels. Exposure of islets to these agents leads to rapid inhibition of insulin release [8]. As shown in Figure 2 glucose-induced MTT reduction was impaired by pre-exposure of INS-1 cells to streptozotocin in a dose-dependent manner. Under these experimental conditions, 3 and 10 mmol/l streptozotocin completely abolished the glucose effect while a small inhibition was already seen at 1 mmol/l. The results of three separate experiments were: glucose 16.7 mmol/l = 144.7 ± 9.6, glucose 16.7 mmol/l + streptozotocin 10 mmol/l = 109.9 ± 13.2 (optical density as % of basal, mean ± SEM, $p < 0.025$). Similar experiments were performed in which INS-1 cells were pre-incubated (15–60 min) in the presence of alloxan 2 mmol/l in a glucose-free KRB, and subsequently incubated (120 min) either in the absence or the presence (33.4 mmol/l) of glucose. The results of three separate experiments each performed with five replicas (mean ± SEM) are given as follows: optical density as %; glucose 0 mmol/l = 100 ± 23, glucose 33.4 mmol/l = 151 ± 38, glucose 0 mmol/l + alloxan = 56 ± 8, glucose 33.4 mmol/l + alloxan = 54 ± 15. Alloxan thus strongly inhibited the formazan production at both glucose concentrations ($p < 0.05$ for glucose 0 mmol/l and $p < 0.02$ for glucose 33.4 mmol/l). As shown in Figure 2, in INS-1 cells, the half-maximal effect of alloxan was found to be 2.0 mmol/l.

In a limited series of experiments, the effect of streptozotocin (1 mmol/l) and alloxan (1 mmol/l) were also

tested in islets: optical density % basal; mean ± SEM; glucose 2.8 mmol/l = 100 ± 37 (n = 7), glucose 16.7 mmol/l = 187 ± 33 (n = 7), glucose 16.7 mmol/l + streptozotocin = 43 ± 12 (n = 4), glucose 16.7 mmol/l + alloxan = 51 ± 14 (n = 7). In these experiments the cytotoxic agents were present during the 30-min pre-incubation (glucose 2.8 mmol/l) as well as during the 60-min subsequent exposure to MTT at both high or low glucose concentrations. In contrast to INS-1 cells, alloxan and streptozotocin did not lower formazan production in RINm5F cells under the same experimental conditions as those used in Figure 2 (not shown). This is in agreement with the reported relative lack of effect of these drugs on glucose metabolism in RINm5F cells [7].

The reduction of MTT has been suggested to reflect indirectly mitochondrial oxidative processes of living cells [1, 2]. The exact mechanism by which the tetrazolium salts are transformed to formazan crystals inside the cells is only partially understood. Although the transfer of electrons from the mitochondrial succinate dehydrogenase complex has been suggested to be coupled with formazan production, other dehydrogenases may also be effective in reducing the tetrazolium salt [9, 10]. The experiments performed on islets indicate that the rate of MTT reduction does not reflect the final step in oxidative metabolism since formazan production increased less as a function of glucose concentration than glucose oxidation. An alternative explanation would be that MTT uptake by the cells is a rate limiting step.

In conclusion, the correlation between glucose metabolism and formazan production in three insulin secreting cell preparations, displaying different kinetics of glucose metabolism, validates the MTT test as a rapid, simple and convenient means of measuring Beta-cell function. Possible applications of this sensitive test in diabetes research include the screening for Beta cytotoxic agents and its use in research on Beta-cell death, and the on-line evaluation of islet cell preparations in islet transplantation studies with potential clinical implications.

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