Overexpression of lactate dehydrogenase A attenuates glucose-induced insulin secretion in stable MIN-6 β-cell lines

Chao Zhao, Guy A. Rutter*

Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD, UK

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Abstract Since islet β-cells express little L-lactate dehydrogenase (LDH) activity, we have examined the effects on these cells of LDH overexpression. In mock-transfected MIN6 β-cells, LDH activity was 38 nmol/min/mg protein, and 30 mM glucose stimulated secretion 10.4-fold. In two MIN6 cell clones stably overexpressing human LDH-A cDNA, insulin secretion was stimulated only 2.7- and 2.1-fold by high glucose. K^+-stimulated insulin secretion was unaffected, and leucine stimulation enhanced, by LDH-A overexpression. LDH-A-overexpressing clones displayed unaltered activities of hexokinase, glucokinase, and malate dehydrogenase, slightly elevated plasma membrane lactate transport activity, and lowered insulin content. Low LDH activity would therefore appear important in β-cell glucose sensing.

Key words: Islet β-cell; Lactate dehydrogenase; Insulin secretion; MIN6

1. Introduction

Pancreatic islet β-cells play an important role in sensing physiological variations of extracellular glucose concentration and exert precise regulation by secreting insulin [1]. Glucose and other nutrients stimulate insulin secretion through their metabolism, which probably produces increases in the intracellular concentration of ATP and other metabolic intermediates [2]. Pancreatic islet β-cells and the differentiated INS-1 β-cell line are characterised by very low activities of L-lactate dehydrogenase (LDH) and plasma membrane lactate transport. In contrast, the activity of the FAD-linked mitochondrial glycerol phosphate dehydrogenase ($GPDH_m$), a key enzyme in glycerol phosphate shunt, is much higher in these cells than in islet non-β-cells, liver cells and poorly differentiated islet β-cells lines [3,4]. The level of these two enzymes may therefore be important in sensing changes in the concentration of glucose and other nutrients, by favouring mitochondrial metabolism [3]. There is evidence from rat models of type II diabetes [5] and streptozotocin-induced diabetes [6], that maintained high levels of $GPDH_m$ are important to ensure the proper regulation of insulin secretion by glucose. By contrast, the role of low LDH activity in glucose-stimulated insulin secretion is unknown, though it is predicted that in conjunction with the abundance of $GPDH_m$, it contributes to the channelling the glycolytic pyruvate and NADH towards mitochondrial oxidation [3]. Thus, a much higher proportion of glucose is fully oxidised to CO_2 and H_2O in INS-1 cells compared with the undifferentiated islet beta tumour cell line, RINm5F [3]. The latter line shows a several hundred-fold higher ratio of LDH:$GPDH_m$ activities as well as an impaired pattern of glucose-stimulated insulin secretion [3].

Here, we demonstrate that cells of another glucose-responsive and well-differentiated β-cell line, MIN6 [7], shows low levels of lactate dehydrogenase and plasma membrane lactate transport activity. We have used this cell type to investigate the role of low LDH levels by establishing stable lines stably overexpressing cDNA encoding the A (muscle) isoform of LDH, the only detectable LDH isoform in purified primary islet β-cells [3]. Graded overexpression of LDH-A is shown to ablate the ability of glucose to stimulate insulin secretion. Maintenance of low LDH levels would therefore appear important to ensure proper regulation by glucose of insulin release, and could potentially contribute to the loss of β-cell glucose responsiveness in some forms of non-insulin-dependent diabetes mellitus.

2. Materials and methods

2.1. Cell culture and establishment of stable lines

MIN6 cells [7] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM D-glucose (Sigma), supplemented with 15% heat-inactivated foetal bovine serum (Gibco), 50 μM mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate, in 37°C, 95% air/5% CO_2 incubator. To prepare stable cell lines overexpressing LDH-A, cDNA encoding human LDH-A [8] (kindly provided by Dr S.S. Li, NIH, Research Triangle Park, NC) was subcloned into the mammalian expression vector pcDNA3 via flanking *Eco*RI and *Not*I sites, to allow constitutive expression under cytomegalovirus immediate early gene promoter control. The resultant plasmid (pCMV.LDHA) was introduced into MIN6 cells (passage 18) using the lipoamine, Transfectam (Promega) (6 μg DNA:19 μl Transfectam per 25 cm^2 flask). Stably transfected cells were selected for 3–4 weeks in medium containing 500 μg/ml G418 (Sigma). G418-resistant clones were isolated and expanded in the same medium. Stable clones incorporating only the pcDNA3 backbone ('mock-transfected') were isolated in identical fashion. Assay of enzyme activities was performed in parallel with insulin secretion for each passage examined. INS-1 cells were cultured in RPMI exactly as described [3].

2.2. Assay of enzyme activities

The activity of LDH and other enzymes in extracts of MIN6 and INS-1 cells was determined as described previously [3] with slight modification. Briefly, cells cultured in 25 cm^2 flasks were released with trypsin and washed twice with ice-cold phosphate-buffered saline (PBS). Cell extract was prepared in 0.1 M KH_2PO_4, pH7.2, 2 mM EDTA, 1 mM dithiothreitol by three freeze/thaw cycles. Protein content was measured by Bradford's assay [9] with bovine serum albumin (BSA; Boehringer, grade V) as standard. Consumption or production of NADH were monitored at 37°C by following changes in absorbance at 340 nm with a Unicam UV4 spectrophotometer, with data collection through an on-line PC (Unicam software).

*Corresponding author. Fax: (44) (117) 928-8274.
E-mail: g.a.rutter@bris.ac.uk

Abbreviations: LDH, lactate dehydrogenase; $GPDH_m$, mitochondrial glycerol phosphate dehydrogenase; KRBH, Krebs-Ringer-bicarbonate-HEPES medium; pH_i, intracellular pH

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2.3. Measurement of intracellular pH and plasma membrane lactate transport activity

Intracellular pH (pH_i) and lactate transport activity were measured by monitoring fluorescence changes at the single cell level using 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as an intracellular pH indicator [10]. Briefly, cells grown on glass coverslips and preloaded with BCECF for 20 min were perifused continuously at a flow rate of 2 ml/min on the stage of a Nikon Diaphot microscope equipped with a 40× oil immersion objective. The ratio of the emitted light at two excitation wave lengths (440/490 nm) was used to monitor pH_i, using commercially available software (Cairn Instruments, Faversham, Kent, UK) for data acquisition. The effects of glucose on pH_i were measured at 37°C in Krebs-Ringer bicarbonate medium (KRBH, comprising 140 M NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, pH 7.4 and 0.1% (w/v) bovine serum albumin). Plasma membrane lactate transport activity was measured at 20°C in HEPES-buffered saline containing 150 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 3.3 mM MOPS and 10 mM HEPES (pH 7.4).

2.4. Insulin secretion

Static incubation was used for insulin secretion studies. Cells were seeded in 96 well culture plates (Costar, Cambridge, MA) at a density of 3×10^4 per well and cultured for 3 days. The attached cells were washed twice with glucose-free KRBH (Section 2.3). Where a high concentration of KCl was used, equimolar NaCl was removed to maintain osmolality. Cells then were pre-incubated at 37°C for 60 min in the same buffer, followed by another 60 min during which the buffer was replaced with KRBH containing stimulatory agents. Samples were taken for measuring insulin release and frozen at −20°C until assay. Cellular DNA from each clone was extracted and measured by a fluorescent method using the dye Hoechst 33258 [3], with calf thymus DNA (Sigma) as standard. Released insulin and the cellular insulin content of different clones were determined by double antibody plus polyethyleneglycol radioimmunoassay (Linco Res., St. Louis, MO) with rat insulin as standard.

2.5. Data analysis

All data were expressed as mean ± standard error. Unpaired Student's *t*-test was used to determine the significance where $P < 0.05$.

3. Results

3.1. Enzymatic characterisation of normal and LDH-A-overexpressing MIN6 cell lines

MIN6 cells used in this study (passages 18–26) displayed low LDH levels, at 38.9 ± 13.3 nmol/min/mg protein ($n = 9$ separate cultures) compared with 14.5 ± 3.4 ($n = 3$) for INS-1 cells. The latter value is comparable to previous measurements based on fluorimetric assay of LDH in INS-1 cells [3]. Thus, MIN6 cells expressed considerably lower LDH levels than poorly differentiated islet β-cell lines such as HIT-T15 and RINm5F cells, islet non-β-cells, and liver cells [3]. However, we noted that there was a gradual increase in LDH activity with increasing MIN6 cell passage number (beyond passage 26) in parallel with decreases in secretory responses to glucose (data not shown). Stable cell lines transfected with only pcDNA3 (mock-transfected, A) showed similar LDH activity (34.7 ± 7.5 nmol/min/mg protein, $n = 9$) to untransfected cells of the same passage number. By contrast, in two clones (B and C) of pCMV.LDHA-transfected stable MIN6 cells, the LDH activity was increased by 3.5- and 4.9- fold to 123.4 ± 14.8 ($n = 11$) and 170.6 ± 39.2 nmol/min/mg protein ($n = 14$).

Small differences were also apparent between individual clones in glucose phosphorylation rate observed in cell extracts either at low (0.5 mM) or high (40 mM) glucose. In particular, low-K_m hexokinase activity represented a higher

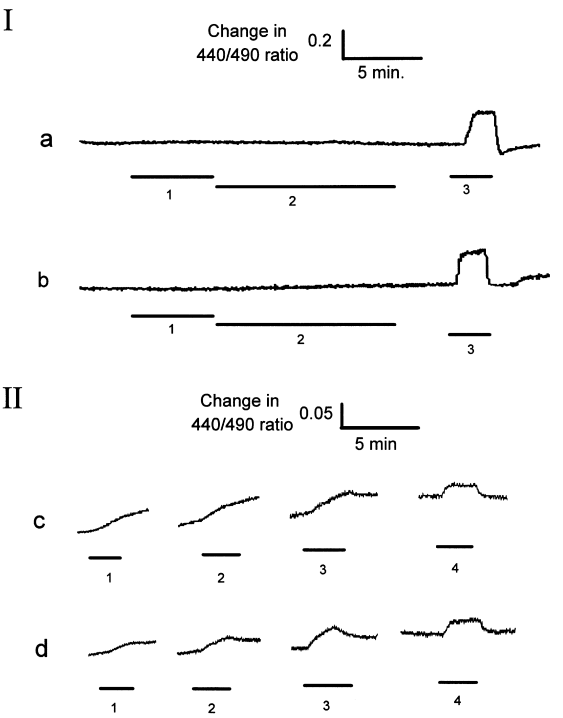

Fig. 1. Effects of glucose on intracellular pH (pH_i) changes (I) and lactate transport activity (II) on MIN6 cells stably transfected with pcDNA3 (a and c) and pCMV.LDHA (b and d; clone C). Panel I: traces a and b show changes in the ratio of fluorescence at 440:490 nm in the absence and presence of glucose. Horizontal bars under each trace indicate the duration of superfusion with the different agents. Bars 1–3 represent 3 mM glucose, 30 mM glucose and 2 mM sodium butyrate. In panel II, traces c and d show the pH_i changes when cells were superfused with different concentrations of sodium lactate. Bars 1–4 represent 1, 5 and 20 mM sodium lactate, and 2 mM sodium butyrate, respectively. This concentration of butyrate, a cell-permeant weak acid, is predicted to caused approximately a 0.1 unit increase in pH_i [10]. All traces are representative of 3–4 separate experiments.

Table 1
Glucose-phosphorylating activity, malate dehydrogenase (MDH) and cellular insulin content in normal and LDH-transfected MIN6 β-cells

	Normal MIN6 (passage 20)	A (mock)	B (low expression)	C (high expression)
Rate of glucose phosphorylation at:				
0.5 mM	4.10 ± 1.27 (2)	1.39 ± 0.35 (6)	0.54 ± 0.19 (3)	0.87 ± 0.21 (6)
40 mM	8.97 ± 2.87 (2)	5.79 ± 1.76 (6)	4.88 ± 1.75 (3)	4.19 ± 1.37 (6)
Ratio (0.5 mM/40 mM)	0.45 ± 0.2	0.24 ± 0.07	0.11 ± 0.05	0.21 ± 0.07
MDH	1310	1120	1182	1063
Insulin content (ng/10^6 cells)	192.4 ± 26.6 (5)[a]	140.9 ± 4.9 (6)[b]	105.4 ± 8.4 (6)[c]	81.8 ± 4.9 (6)[d]

Data are expressed as nmol NADH/mg protein/min and represent duplicate determinations on cells from the number of separate cultures indicated. a vs b, b vs c and b vs d: $P < 0.01$, a vs b: $P < 0.05$.

proportion of total glucose phosphorylating activity in the mock-transfected cells than in either of the LDH-A-overexpressing clones. Nevertheless, the total glucose phosphorylating activity of each clone (i.e. that measured in the presence of 40 mM glucose) was essentially identical (Table 1). Similarly, the total cellular content of malate dehydrogenase was unaltered in the isolated clones.

3.2. Effect of glucose on pH_i and lactate transport activity in control and LDH-A-overexpressing MIN6 cells

As shown in Fig. 1a,b, continuous perfusion with 3 mM glucose for 5 min, followed by 30 mM glucose for 10 min, caused little change in pH_i in either mock- or LDH-A-transfected cells (clone C, above). In each case, perifusion of cells in the presence of 3 or 30 mM glucose increased the fluorescence ratio of BCECF by less than 1% of the initial value, corresponding to an acidification of <0.015 pH units. Furthermore, overexpression of LDH-A caused no apparent increase in the rate of transport of lactate at 1 or 5 mM lactate, although a small increase ($<20\%$) in activity was apparent at 20 mM lactate (Fig. 1c,d). It should be noted that the measured lactate transport activity of MIN6 cells measured in this study was much lower than that of other cell types, including cardiac myocytes [10], but similar to that of primary β-cells and other β-cell-derived lines [3].

3.3. Effects of LDH-A overexpression on insulin content and secretion

MIN6 cells transfected with pcDNA3 (mock-transfected, group A) and two clones overexpressing LDH-A (groups B and C) were used to measure insulin content and glucose-stimulated insulin secretion. As shown in Table 1, the cellular insulin content of each of the stably transfected cell lines was lower than that in normal low passage MIN6 cells. Furthermore, the insulin content of the LDH-overexpressing cells was significantly lower than that of the mock-transfected line.

As illustrated in Fig. 2, for all three groups, incubation for

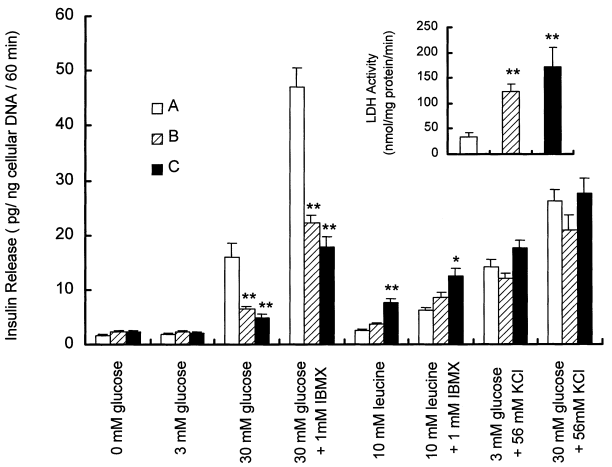

Fig. 2. Stimulated insulin release from MIN6 lines expressing differing levels of lactate dehydrogenase (LDH-A) activity. Insulin release from three MIN6 cell clones (A, B and C) expressing different levels of LDH-A was determined by radioimmunoassay. Cells were stimulated with various agents as shown for 60 min. Data represent the means of release observed in six separate cultures. A: Cells transfected with plasmid pcDNA3 alone. B and C: Cells transfected with pCMV.LDHA. LDH activities are given in the inset (see text). Mean ± S.E.M., $*P<0.05$, $**P<0.01$, compared with group A.

60 min at 3 mM glucose elicited no significant increase in insulin release compared with incubation at 0 mM glucose. By contrast, when incubated in the presence of 30 mM glucose, group A showed a 10.4 ± 1.6-fold ($n=6$ separate cultures) increase in insulin release compared to the release observed from cells incubated in the absence of glucose. In groups B and C, this increment was reduced to 2.7 ± 0.2- and 2.1 ± 0.4-fold, respectively. Incubation of cells in the additional presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) further enhanced insulin secretion (Fig. 2). Nonetheless, the relative rates of insulin release from the different groups was similar to that in incubations performed at 30 mM glucose alone. These data indicated that the insulin release induced by glucose was attenuated by overexpression of LDH-A. Leucine (10 mM) caused a 1.69 ± 0.11-fold ($P<0.05$, $n=6$) increase in insulin release in group A. Whereas a similar increase in insulin release in response to leucine was observed in group B, the effect of leucine in group C was enhanced to 3.34 ± 0.30-fold. Similarly, when cells from each clone were stimulated with KRBH containing 56 mM KCl, in the presence of either 3 or 30 mM glucose, the elevation of insulin secretion showed no difference among the three groups (Fig. 2).

4. Discussion

These data indicate that MIN6 cells, like primary islet β- and INS-1 cells, express low levels of LDH activity and display near-normal glucose-stimulated insulin release. Combined with the recent finding that a further glucose-sensitive β-cell line, β-HC9 cells, express low LDH levels [4] the data would appear further to strengthen the link between low LDH levels in β-cells and normal glucose-stimulated insulin release. In addition to this compelling, but correlative evidence, we now demonstrate that molecular manipulation of LDH levels in the tractable MIN6 β-cell line leads to alterations in the extent of glucose-stimulated insulin secretion. Thus, stably transfected MIN6 cells displayed dramatically increased LDH activity but otherwise similar activities of a number of other key enzymes believed to be important for glucose sensing, including hexokinase and glucokinase, and lactate transport activity. However, LDH-A-transfected cells showed markedly reduced secretory responses to glucose, but unaltered or enhanced responses to other secretagogues. In addition, the LDH-A-overexpressing clones also expressed significantly lower levels of insulin. This indicates that the generation by external glucose of signals responsible for maintained transcription or translation of the insulin gene may also depend on low LDH activity.

Overexpression of LDH is expected to divert glucose carbon (as pyruvate) and glycolytic reducing equivalents, from mitochondrial oxidation. We [3] and others [4,11,12] have proposed that mitochondrial oxidation is essential for glucose-stimulated insulin secretion, either to ensure adequate ATP supply or to ensure anaplerosis ('topping up') of the citrate cycle. The latter may be important both for amino acid biosynthesis and malonyl CoA production, the latter acting as a coupling signal for secretion [2]. It is unlikely that the differences in of glucose-induced insulin secretion between LDH-A-transfected and control cells in this study was due to alterations in the intracellular accumulation of lactate. Thus, with either control or transfected cells, glucose-induced changes in intracellular pH were small if apparent at all,

under our experimental conditions. These observations are compatible with previous measurements of glucose-induced changes in pH_i in β-cells and derived lines, which have indicated that these changes are small, and highly dependent on HCO_3^- concentration [13]. Thus, the predicted increase in lactate production by the LDH-expressing cells would appear not to result in a significant intracellular accumulation of lactate. In part, this may be related to a small increase in plasma membrane lactate transport activity detected in the stably transfected lines at high lactate concentrations (Fig. 1). However, we have found that rates of production of lactate by both transfected and non-transfected MIN6 cell lines, examined as described earlier in INS-1 cells [3], are low and close to the limit of detection (C. Zhao, G.A. Rutter and J. Tamarit-Rodriguez, unpublished data).

Recently, others [14] have confirmed our own [3] measurements of low LDH activity (about 80 nmol/min/mg protein) in FACS-purified islet β-cells. These authors have stressed that, although many orders of magnitude lower than in other (non islet) cell types, the V_{max} of LDH in β-cells is well in excess of that of glucose-phosphorylating enzymes. It should be noted, however, that flux through the enzyme will be a function of other kinetic parameters, notably the K_m of the enzyme compared to competing enzymes (including the mitochondrial pyruvate carrier), and the concentration of substrates (pyruvate and NADH). Therefore, an increase in the V_{max} of the enzyme, achieved in the present studies by overexpression of recombinant human LDH-A, would still be expected to alter the relative fluxes through LDH versus mitochondrial oxidation. Furthermore, the activation of oxidative metabolism at high glucose by Ca^{2+} stimulation of mitochondrial glycerol phosphate dehydrogenase and intramitochondrial dehydrogenases [15,16] is clearly entirely compatible with, and complementary to, the absence of high levels of LDH in this cell type. However, Jijakli et al. [14] have suggested that the ratio of LDH:$GPDH_m$ activity is closely similar in islet β- and islet non-β-cells. This observation is surprising, and contrary to our own observations based on the quantitation of $GPDH_m$ in non-β-cells by immunoblotting [3].

Deranged β-cell LDH expression in vivo, mimicked in the present in vitro studies, is therefore likely to provoke the misregulation of insulin release, and could in principle contribute to the glycaemia observed in non-insulin-dependent diabetes mellitus. Studies of diabetic models, and genetic linkage analysis to the human disease, will be required to test this hypothesis. At the same time, manipulation of β-cell LDH levels might represent a potential therapeutic target for this condition.

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