Importance of lactate dehydrogenase for the regulation of glycolytic flux and insulin secretion in insulin-producing cells

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The role of lactate dehydrogenase (LDH) in the generation of the metabolic signal for insulin secretion was studied after stable overexpression in INS-1 and RINm5F insulin-producing cells. INS-1 cells with a 25-fold overexpression of LDH-A, the highest level achieved, showed a 20–30% decrease in the glucose oxidation rate at glucose concentrations above 5 mM when compared with control cells, whereas values were unchanged at lower glucose concentrations. Lactate release increased in parallel with a decrease in the glucose oxidation rate. However, the INS-1 cell glucose-induced insulin secretory response, together with the rate of glucose utilization, were not significantly affected by LDH-A overexpression. Despite 3-fold overexpression of LDH-A in glucose-unresponsive RINm5F cells, there was no change in insulin secretion, glucose metabolism or lactate production in these cells. Exogenously added pyruvate and lactate potentiated glucose-stimulated insulin secretion in INS-1 cells, an effect that was abolished after LDH-A overexpression. Both compounds significantly decreased glucose oxidation rates in control cells. After overexpression of LDH-A in INS-1 cells, the effects of pyruvate and lactate on glucose oxidation were diminished. On the other hand, after LDH-A overexpression, both glycolytic metabolites decreased the glucose utilization rate at 5 mM glucose. The present data suggest that the level of LDH expression in insulin-secreting cells is critical for correct channelling of pyruvate towards mitochondrial metabolism. Interestingly, glucokinase-mediated glycolytic flux was decreased after LDH-A overexpression. Thus preferential channelling of glucose towards aerobic metabolism by glucokinase may be determined, at least in part, by the low level of constitutive expression of LDH-A in pancreatic β-cells. In conclusion, the level of LDH expression in insulin-secreting cells is an important determinant of the physiological insulin-secretory capacity, and also determines how pyruvate and lactate affect insulin secretion.

Key words: clonal insulin-secreting cell lines, glucose metabolism, INS-1 cells, overexpression, RINm5F cells.

INTRODUCTION

It is generally accepted that the glucose-induced release of insulin by pancreatic β-cells is a metabolically driven process [1–5]. Metabolic rates for this hexose correlate well with its insulinotropic potency, and it has been suggested that the generation of metabolic signals, such as an increase in the ATP/ADP ratio, is critical for insulin stimulus–secretion coupling [6]. Thus the partitioning of glucose metabolic flux between glycolysis and mitochondrial oxidation has been regarded as an important regulatory element, since it has been assumed that the insulin-secretory capacity of glucose is dependent on signals generated during aerobic metabolism [7]. Such a hypothesis is supported in particular by the preferential stimulation of aerobic metabolism in islet cells in the presence of millimolar concentrations of extracellular glucose [8–10]. Conversely, there is experimental evidence that glycolytically, rather than mitochondrially, generated ATP might be crucial for closure of K_{ATP} channels, which in turn may regulate fuel-mediated insulin secretion [11]. In addition, it has been proposed that high mitochondrial glycerol phosphate dehydrogenase activity in combination with low lactate dehydrogenase (LDH) activity may channel glycolytic pyruvate preferentially into mitochondrial oxidation [12].

LDH (EC 1.1.1.27) is the enzyme involved in the final step of anaerobic glycolysis. Various tetrameric LDH isoenzymes are expressed in different tissues, and are composed of LDH-A (so-called muscle type) and LDH-B (so-called heart type) subunits. Of these, only the A-type is expressed in purified pancreatic β-cells [12]. To evaluate a putative role of LDH isoenzymes in the regulation of the aerobic and anaerobic partition of glucose metabolic flux, we overexpressed LDH-A in insulin-secreting cell lines. It has been shown previously that LDH overexpression in a cell line with low LDH activity (MIN-6 cells) resulted in a decrease in glucose-induced insulin secretion [13]. Contradictory results were obtained more recently in another study in which glucose-induced insulin secretion was not affected after overexpression of LDH and/or the monocarboxylate transporter in INS-1 cells [14].

The role of LDH in the regulation of glucose metabolism is determined by various factors, such as the absolute activity level of LDH, the glycolytic flux initiated by glucokinase/hexokinase and the complex process of channelling of glycolytic products to aerobic mitochondrial metabolism. In particular, the relationship between the anaerobic and aerobic branches is important in this context. We therefore investigated this question in the present study in insulin-secreting INS-1 cells after the graded overexpression of LDH with respect to glucose metabolism and insulin-secretory capacity. Such an approach might also allow a better understanding of the well known lack of insulin-secretory potency of pyruvate [15–18] and lactate [7,19,20], which are poor substrates for the pancreatic β-cell [7,16,18,21–24].

In order to clarify the interaction of glycolytic flux initiated by glucokinase and by hexokinase with the function of LDH for the partitioning of aerobic and anaerobic glucose metabolism, we

Abbreviation used: LDH, lactate dehydrogenase.

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used both the cellular models of the INS-1 and RINm5F insulin-producing permanent tissue culture cell lines. While INS-1 cells have a low LDH activity with a glucokinase-dominated glycolytic flux, RINm5F cells have a high LDH activity with a hexokinase-dominated glycolytic flux. Thus overexpression of LDH in these cell lines provides an approach to elucidating the distinct interrelationships between LDH and the low-affinity glucokinase component as well as the high-affinity hexokinase component of glucose metabolism.

In the present study we show that a low level of LDH-A activity in insulin-secreting cells is crucial in order to ensure the preferential channelling of glucose-derived pyruvate towards mitochondrial oxidation, so as to preserve glucose-induced insulin-secretory responsiveness and to suppress unwanted stimulation of insulin secretion by pyruvate and lactate.

MATERIALS AND METHODS

Materials

Restriction enzymes, the SP6/T7 Transcription Kit and the digoxigenin Nucleic Acid Detection Kit were obtained from Boehringer (Mannheim, Germany). Hybond N nylon membranes and autoradiography films were from Amersham (Braunschweig, Germany). D-[U-^{14}C]Glucose and D-[5-^{3}H]glucose were from Hartmann (Braunschweig, Germany). Geneticin (G418), LIPOFECTAMINE™ and all other tissue culture equipment were from GIBCO Life Technologies (Gaithersburg, MD, U.S.A.). Guanidine thiocyanate was from Fluka (Neu-Ulm, Germany). ^{125}I-labelled insulin was kindly provided by Hoechst (Frankfurt, Germany). All other reagents were of analytical grade and were from Merck (Darmstadt, Germany). The INS-1 cell line was kindly provided by Dr M. Asfari (INSERM, Paris, France). The RINm5F rat cell line was from the American Type Culture Collection (A. T. C. C.; Rockville, MD, U.S.A.).

Stable overexpression of LDH in INS-1 and RINm5F cells

INS-1 cells (passage no. 80–90) [25] were grown in RPMI 1640 tissue culture medium, supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 50 μM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. RINm5F cells (passage no. 80–90) [26] were grown in RPMI 1640 tissue culture medium, supplemented with 10 % (v/v) foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cDNA of the A-isoform of rat LDH was synthesized by reverse transcriptase–PCR with rat muscle RNA by using primers (forward, ATG GCA ACT CTA AAG GAT CA; reverse, TTA AAA TTG CAG CTC CTT TT) which were constructed based on the published sequence of the mRNA for rat LDH (GenBank® accession no. X01964). This cDNA, under the transcriptional control of the cytomegalovirus promoter, was subcloned into the pcDNA3 expression vector (Invitrogen, Leek, The Netherlands) using standard molecular biology techniques [27,28]. INS-1 and RINm5F cells were transfected using LIPOFECTAMINE™. Positive clones were selected by geneticin (G418) resistance and characterized further for LDH expression by Northern blot analyses and measurement of enzymic activity. The INS-1 and RINm5F clones with the highest stable levels of expression of LDH were selected for detailed biochemical characterization. Unless otherwise indicated, experiments were performed in 20 mmol/l Hepes-buffered Krebs/Ringer bicarbonate medium (pH 7.4) [20]. The DNA content of cells was determined according to [29].

Northern blot analyses

Cells were lysed in pre-cooled buffered 4 mol/l guanidine thiocyanate solution. Total RNA was isolated by a combined water-saturated phenol/chloroform/3-methylbutan-1-ol extraction method [30]. A total of 20 μg of RNA per lane was subjected to electrophoresis on denaturing formamide/formaldehyde 1 % (w/v) agarose gels and transferred to nylon membranes. Hybridization was performed at 68 °C overnight in a solution containing 50 % deionized formamide, 5 × SSPE (1 × SSPE = 150 mmol/l NaCl, 1 mmol/l EDTA and 10 mmol/l NaH_2PO_4, pH 7.4), 10 × Denhardt's solution (1 × Denhardt's = 0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone and 0.002 % BSA), 0.5 % SDS, 100 μg/ml sonicated non-homologous DNA from herring sperm and 11-digoxigenin-UTP-labelled antisense cRNA probes coding for the A-isoform of LDH. The digoxigenin-labelled hybrids were detected by an enzyme-linked immunoassay using an anti-digoxigenin alkaline phosphatase antibody conjugate and subsequent enzyme-catalysed chemiluminescent detection with the substrate CSPD® [3-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate].

LDH assay

Total LDH enzyme activity was measured in sonicated tissue homogenates by a photometric assay [31]. Cells were pelleted by gentle centrifugation (400 ***g***, 5 min), resuspended in 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7) and lysed by sonication (60 W; three bursts of 10 s). A typical assay consisted of 0.87 ml of 0.1 M phosphate buffer (pH 7.35), 50 μl of 0.5 M sodium pyruvate and 20 μl of 10 mM NADH, in a final volume of 945 μl. In all cases the reaction was initiated by the addition of enzyme, and was monitored by the decrease in absorbance at 340 nm. Enzyme activity was expressed as units/mg of protein. One unit of enzyme activity was defined as the transformation of 1 μmol of pyruvate per min at 30 °C. Protein was determined by a BCA assay (Pierce).

Lactate production

Lactate production was measured in the incubation medium by a photometric assay containing LDH, glutamate–pyruvate transaminase and NAD^+ [32]. Large batches of cells (5×10^6) were incubated for 60 min at 37 °C in Krebs/Ringer buffer containing different glucose concentrations. Media were collected, centrifuged gently (400 ***g***, 5 min) and deproteinized with perchloric acid. Values were expressed as mmol of hexose equivalents/h per g of DNA.

Glucose metabolism

The rates of glucose utilization and oxidation were assessed as the production of ^3H_2O and $^{14}CO_2$ from D-[5-^{3}H]glucose and D-[U-^{14}C]glucose respectively. Glucose metabolism was measured as described [33] in batches of 1.5×10^5 cells over a 1 h incubation at 37 °C in 40 μl of Krebs/Ringer buffer containing various glucose concentrations. Total radioactivity added to the cells was 10 μCi/ml, resulting in specific radioactivities of 0.2–40 Ci/mol. Cellular metabolism was stopped by the addition of 50 μl of 0.2 M HCl. 1-Phenylethylamine was used to capture the produced $^{14}CO_2$, and water was used for ^3H_2O. Recoveries of externally added $NaH^{14}CO_3$ and ^3H_2O were checked routinely and used to correct the metabolic rates accordingly.

Insulin secretion

Insulin released into the medium was assessed using static incubations. Cells were seeded at a density of 1×10^5/well in 12-well culture dishes and grown for 72 h in the appropriate medium supplemented with 10 mmol/l glucose. Cells were then washed twice with glucose-free medium and preincubated with glucose-free medium for 2×15 min at 37 °C. Insulin secretion during a 30 min incubation period was measured in Krebs/Ringer buffer containing different glucose concentrations. After incubation, buffer was removed, centrifuged gently to remove detached cells, and stored at −20 °C for radioimmunological insulin determination using a rat insulin standard.

Statistical analyses

Data are expressed as means ± S.E.M. Statistical analyses were performed using Student's *t*-test.

RESULTS

Stable overexpression of LDH

After transfection of INS-1 and RINm5F cells with the mammalian expression vector pcDNA3-LDH, G418-resistant clones from each cell line were screened by Northern blot analysis for mRNA expression, and LDH activity was determined. Table 1 shows results of the mRNA expression analyses and the LDH enzyme activities for three selected transfected clones of INS-1 and RINm5F cells. In non-transfected INS-1 control cells, LDH mRNA expression was undetectable by Northern blot analysis. The INS-1 clone with the highest level of expression (INS-LDH1) showed an LDH activity approx. 25 times higher than that in non-transfected control cells (Table 1). In addition, one of the other clones (INS-LDH2), showing an intermediate level of overexpression with an enzyme activity 5 times higher than that of the control cells (Table 1), was selected for functional characterization. The three LDH-overexpressing RINm5F clones showed similar mRNA expression levels and LDH enzyme activities. For further functional characterization, the RIN-LDH1 and RIN-LDH2 clones were selected.

Insulin secretion

Non-transfected INS-1 control cells were glucose-responsive at millimolar glucose concentrations. The rate of insulin secretion increased, dependent upon the glucose concentration, by approx. 150%. The half-maximal effective glucose concentration was approx. 3 mM, with maximal responsiveness in the range 5.0–16.7 mM glucose (Figure 1). Overexpression of LDH did not significantly affect the insulin-secretory responsiveness of the INS-LDH1 and INS-LDH2 clones (Figure 1).

Non-transfected RINm5F control cells were not glucose-responsive at millimolar glucose concentrations (Figure 1). LDH overexpression in the RIN-LDH1 and RIN-LDH2 clones did not significantly affect the insulin-secretory pattern (Figure 1). Comparable observations were made with the RIN-LDH3 clone (results not shown). Insulin content was not significantly affected by overexpression of LDH in any of the INS-1 and RINm5F cell clones studied (results not shown).

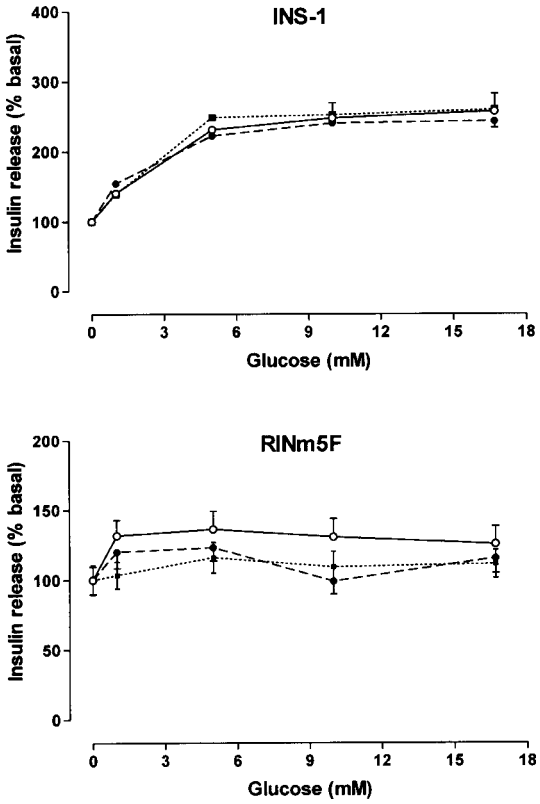

Figure 1 Effects of LDH overexpression on glucose-induced insulin secretion in INS-1 and RINm5F cells

○, Control cells; ●, clones INS-LDH1 and RIN-LDH1; ■, clones INS-LDH2 and RIN-LDH2. Cells were incubated for 30 min at 37 °C in the presence of various glucose concentrations. Insulin was measured in the supernatant, and results were expressed as the rate of insulin secretion above basal. Basal secretory values were (μg/h per g of DNA): INS-1, 146 ± 7 ($n = 20$); INS-LDH1, 168 ± 8 ($n = 11$); INS-LDH2, 140 ± 8 ($n = 8$); RINm5F, 277 ± 15 ($n = 8$); RIN-LDH1, 310 ± 19 ($n = 8$); RIN-LDH2, 313 ± 31 ($n = 8$). Values are means ± S.E.M. from 8–16 experiments.

Table 1 Stable overexpression of LDH in INS-1 and RINm5F cells

Cells were transfected with rat LDH-A cDNA. Overexpressing clones were selected through resistance to G418 and characterized for mRNA expression (by Northern blot) and for enzyme activity. Gene expression of LDH was quantified by densitometric analyses of the autoradiograms. Data are expressed as percentages of the gene expression in RINm5F control cells. Values are means ± S.E.M. for the numbers of independent experiments indicated in parentheses. Significance of differences compared with relevant INS-1 or RINm5F control cells: $^{**}P < 0.01$. N.D., not detected.

Cell clone	1.6 kb mRNA band	mRNA expression (%)	Enzyme activity (units/mg of protein)
INS-1		N.D.	0.4 ± 0.1 (8)
INS-LDH1		303 ± 65** (4)	9.4 ± 0.2** (7)
INS-LDH2		174 ± 40** (4)	2.0 ± 0.1** (6)
INS-LDH3		134 ± 24** (4)	2.4 ± 0.1** (6)
RINm5F		100 ± 00 (4)	6.9 ± 0.4 (8)
RIN-LDH1		332 ± 37** (4)	17.8 ± 0.2** (6)
RIN-LDH2		242 ± 37** (4)	17.8 ± 0.4** (7)
RIN-LDH3		171 ± 30** (4)	15.6 ± 0.2** (7)

Glucose utilization and oxidation

The effect of LDH overexpression on glucose metabolism in INS-1 and RINm5F cells was also investigated. D-[5-^{3}H]Glucose utilization was measured in order to estimate the overall rate of glycolysis. The rate of mitochondrial oxidative metabolism was assessed in studies of D-[U-^{14}C]glucose oxidation.

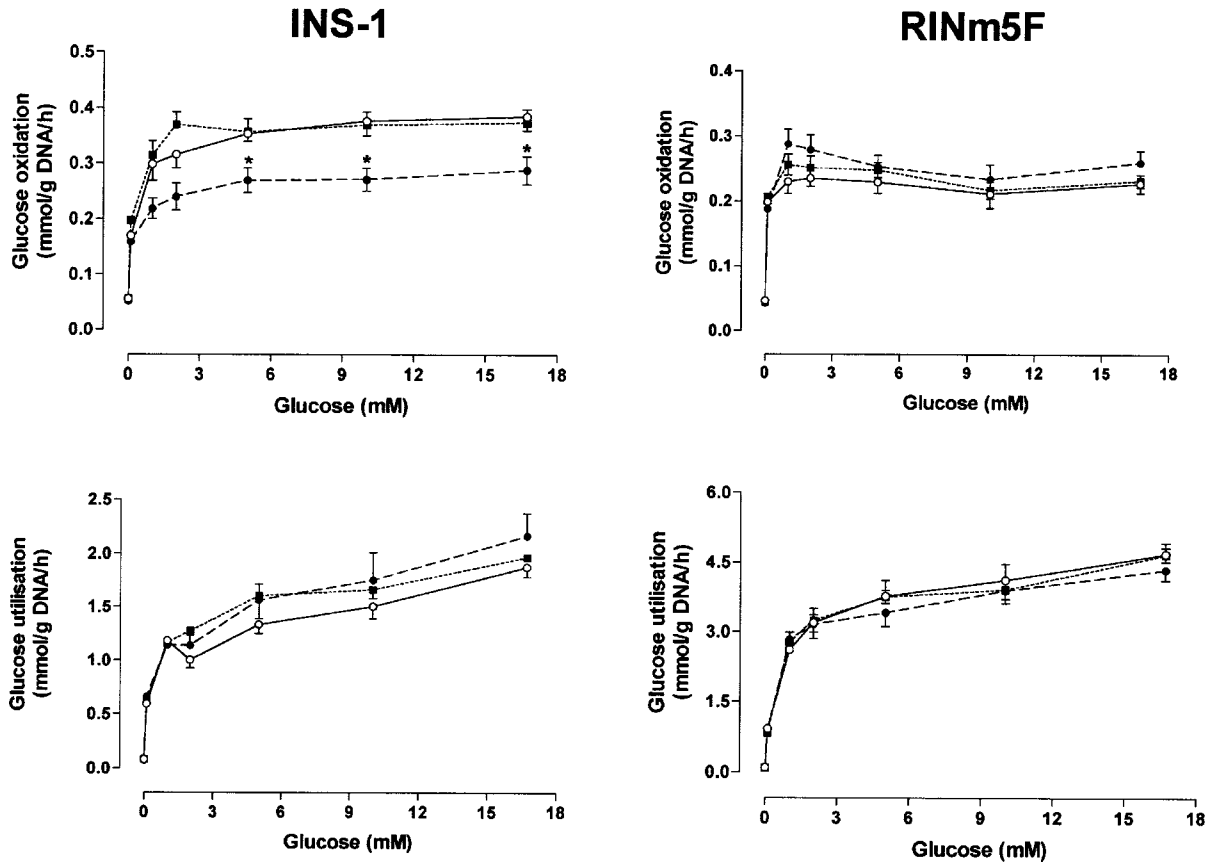

Figure 2 Effects of LDH overexpression on glucose metabolism in INS-1 and RINm5F cells

○, Control cells; ●, clones INS-LDH1 and RIN-LDH1; ■, clones INS-LDH2 and RIN-LDH2. Cells were incubated for 60 min at 37 °C in the presence of various concentrations of D-[U-^{14}C]glucose or D-[5-^{3}H]glucose. Glucose oxidation and utilization rates were calculated from the production of $^{14}CO_2$ and $^{3}H_2O$ respectively. Values are means ± S.E.M. from six independent experiments. Significance of differences: $^{*}P < 0.05$ compared with control cells.

Rates of glucose utilization and oxidation increased in a glucose-concentration-dependent manner in both INS-1 and RINm5F cells (Figure 2). However, only INS-1 cells exhibited glucose-responsiveness at millimolar glucose concentrations. With the exception of clone INS-LDH1, the rates of glucose utilization and oxidation in LDH-overexpressing INS-1 and RINm5F clones did not exhibit significant differences when compared with their respective non-transfected control cells (Figure 2). Unlike the other cells, the INS-LDH1 clone, with the highest level of LDH overexpression, showed a significant decrease in the rate of glucose oxidation at concentrations of 5 mM glucose and above ($P < 0.05$).

Figure 3 shows the calculated ratios for INS-1 cells when the oxidation of D-[U-^{14}C]glucose was expressed relative to D-[5-^{3}H]glucose utilization. In the INS-LDH1 clone, with the highest level of LDH overexpression, the ratio was significantly decreased (Figure 3). Not surprisingly, the decrease was particularly large in the upper range of glucose concentrations (Figure 3), because the decrease in glucose oxidation rate was greatest at these glucose concentrations (Figure 2).

Lactate production

The lactate released into the medium was determined in order to estimate the relationship between the proportions of the glycolytic flux travelling through the aerobic and anaerobic pathways (Figure 4). INS-1 cells overexpressing LDH at a low level (clone INS-LDH2) showed a significant increase ($P < 0.01$) in lactate release only at the highest concentration of glucose tested (16.7 mM) (Figure 4). However, the INS-1 clone with 25-fold LDH overexpression (INS-LDH1) exhibited significantly increased lactate release at all glucose concentrations tested. Again, the effects were greatest at the highest glucose concentrations (Figure 4). Overexpression of LDH in RINm5F cells (RIN-LDH1, RIN-LDH2), on the other hand, did not significantly affect the rate of lactate release.

Effects of pyruvate and lactate on insulin secretion

Rates of insulin secretion, both in the absence and in the presence of 1 and 5 mM glucose, from non-transfected INS-1 cells were increased by pyruvate (10 mM) and lactate (10 mM) by 50–100 % (Table 2). The insulin-secretory effect of methyl pyruvate (10 mM) was even more pronounced in the absence of glucose, while it was comparable with those of pyruvate and lactate in the presence of glucose (Table 2).

Overexpression of LDH (clone INS-LDH1) did not affect the basal rate of insulin release in the absence or in the presence of glucose (1 and 5 mM), but resulted in a significant ($P < 0.01$) decrease in the potentiating effects of pyruvate (10 mM), lactate

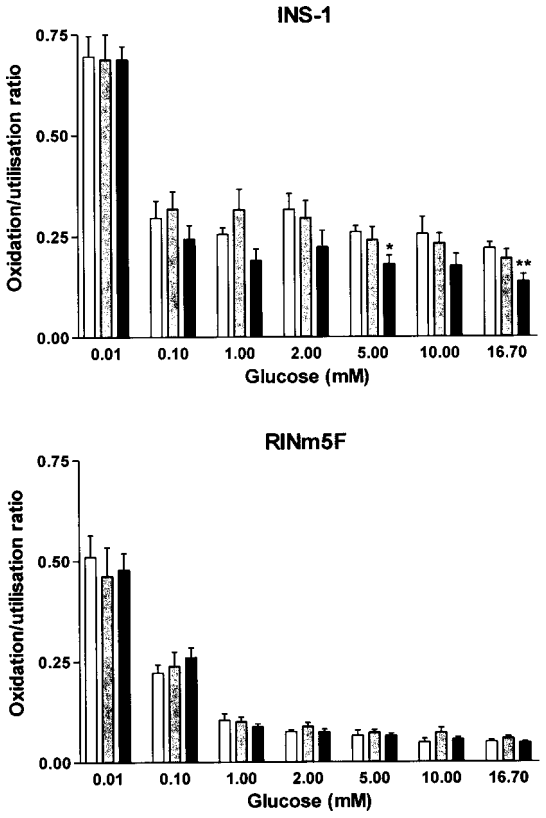

Figure 3 Effects of LDH overexpression on the ratio between D-[U-^{14}C]glucose oxidation and D-[5-^{3}H]glucose utilization in INS-1 and RINm5F cells

Open bars, control cells; black bars, clones INS-LDH1 and RIN-LDH1; grey bars, clones INS-LDH2 and RIN-LDH2. Cells were incubated for 60 min at 37 °C in the presence of various concentrations of D-[U-^{14}C]glucose or D-[5-^{3}H]glucose. Glucose oxidation and utilization rates were calculated from the production of $^{14}CO_2$ and $^{3}H_2O$ respectively. Values are means ± S.E.M. from six independent experiments. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control cells.

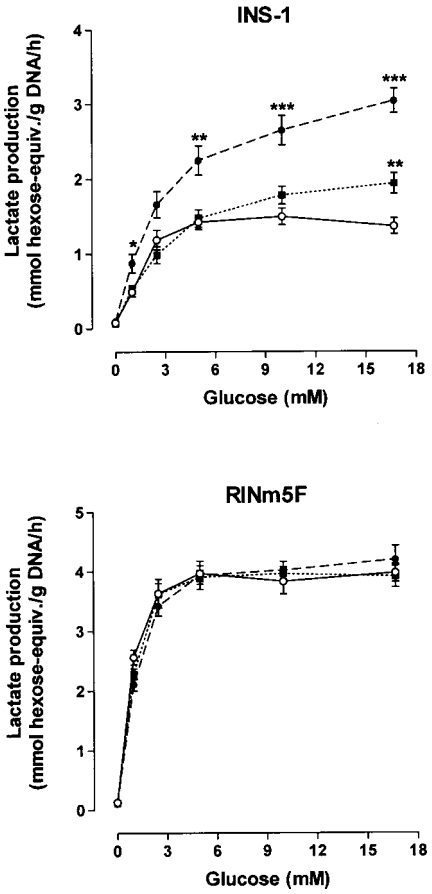

Figure 4 Effects of LDH overexpression on lactate production in INS-1 and RINm5F cells

○, Control cells; ●, clones INS-LDH1 and RIN-LDH1; ■, clones INS-LDH2 and RIN-LDH2. Cells were incubated for 60 min at 37 °C in the presence of various glucose concentrations. Lactate was measured in the incubation medium. Values are means ± S.E.M. from five to six experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with controls.

(10 mM) and methyl pyruvate (10 mM) in the presence, but not in the absence, of glucose (1 and 5 mM) (Table 2).

Effects of pyruvate and lactate on glucose metabolism

Pyruvate (10 mM) and lactate (10 mM) did not affect rates of glucose utilization in non-transfected INS-1 cells in the presence of micromolar (10 μM) or millimolar (1 and 5 mM) glucose concentrations (Table 3). In contrast, methyl pyruvate (10 mM) drastically decreased the rates of glucose utilization at 1 and 5 mM glucose (Table 3).

Cells overexpressing LDH (clone INS-LDH1) showed a significant decrease in the potentiating effects of pyruvate (10 mM) and lactate (10 mM) on glucose utilization in the presence of 5 mM glucose (Table 3). Methyl pyruvate (10 mM), as in non-transfected control cells, had a significant inhibitory effect at all glucose concentrations (Table 3). Compared with non-transfected INS-1 cells, the rates of glucose utilization were not different for any of the compounds used (10 mM).

Pyruvate, lactate and methyl pyruvate (each at 10 mM) drastically decreased the rate of glucose oxidation in non-transfected INS-1 cells in the presence of both micromolar (10 μM) and millimolar (1 and 5 mM) glucose concentrations (Table 4). LDH overexpression (clone INS-LDH1) did not abolish these effects (Table 4).

DISCUSSION

It is well known that an essential feature of pancreatic β-cell metabolism is the preferential stimulation of oxidative, relative to anaerobic, glucose metabolism within the physiological millimolar glucose concentration range [34,35]. A detailed characterization of the regulatory interplay between the aerobic and anaerobic branches of glucose metabolism would help us to understand the underlying mechanisms. In the present study, we overexpressed LDH-A in insulin-secreting cell lines (INS-1 and RINm5F) with different constitutive levels of LDH activity, in order to clarify the importance of this enzyme for coupling of glycolytic and oxidative metabolism. This allowed us to achieve different levels of LDH-A expression, resulting in total LDH enzyme activity 5–25-fold greater than the basal activity (0.4 ± 0.1 unit/mg of protein) due to the constitutive expression of B-type LDH in the INS-1 cell line, which is somewhat higher than that in normal β-cells (0.03 ± 0.00 unit/mg of protein) [12]. The activity of LDH in RIN cells is markedly higher (6.9 ± 0.4 units/mg of protein), consistent with previous reports [12], and was therefore only tripled after LDH overexpression. Unlike

Table 2 Effects of lactate, pyruvate and methyl pyruvate on glucose-induced insulin secretion in non-transfected INS-1 cells and in the LDH-overexpressing cell clone INS-LDH1

Cells were incubated for 30 min at 37 °C in the presence of various glucose concentrations, and the added agents at a concentration of 10 mM. Insulin released into the medium was measured and expressed as μg of insulin/h per g of DNA. Values are means ± S.E.M. for the numbers of independent experiments given in parentheses. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control cells in the presence of the same concentration of glucose alone.

	Insulin secretion (μg/h per g of DNA)	
Additions to medium (mM)	INS-1	INS-LDH1
Glucose (0)	200 ± 17 (8)	241 ± 19 (11)
Glucose (1)	290 ± 38 (8)	298 ± 42 (15)
Glucose (5)	532 ± 58 (8)	479 ± 55 (11)
Glucose (0) + pyruvate (10)	351 ± 17 (8)**	275 ± 25 (15)
Glucose (1) + pyruvate (10)	482 ± 68 (8)*	279 ± 23 (15)
Glucose (5) + pyruvate (10)	858 ± 104 (8)*	408 ± 29 (11)
Glucose (0) + lactate (10)	424 ± 63 (8)**	294 ± 23 (9)
Glucose (1) + lactate (10)	481 ± 33 (8)**	357 ± 38 (11)
Glucose (5) + lactate (10)	980 ± 37 (8)**	554 ± 69 (11)
Glucose (0) + methyl pyruvate (10)	521 ± 53 (8)**	502 ± 22 (11)**
Glucose (1) + methyl pyruvate (10)	576 ± 28 (8)**	419 ± 27 (11)**
Glucose (5) + methyl pyruvate (10)	978 ± 152 (8)*	517 ± 28 (11)

Table 3 Effects of lactate, pyruvate and methyl pyruvate on glucose utilization rates in non-transfected INS-1 cells and in the LDH-overexpressing cell clone INS-LDH1

Cells were incubated for 60 min at 37 °C in the presence of various concentrations of D-[5-^{3}H]glucose, plus the added agents at a concentration of 10 mM. Glucose utilization rates were determined from the production of $^{3}H_2O$. Values are means ± S.E.M. for the numbers of independent experiments given in parentheses. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control cells in the presence of the same concentration of glucose alone.

	Glucose utilization (μmol/h per g of DNA)	
Additions to medium (mM)	INS-1	INS-LDH1
Glucose (0.01)	25 ± 3 (6)	62 ± 12 (6)
Glucose (1)	942 ± 151 (6)	1190 ± 283 (6)
Glucose (5)	1819 ± 101 (6)	2066 ± 293 (6)
Glucose (0.01) + pyruvate (10)	24 ± 1 (6)	57 ± 9 (6)
Glucose (1) + pyruvate (10)	913 ± 188 (6)	741 ± 130 (6)
Glucose (5) + pyruvate (10)	1451 ± 329 (6)	965 ± 221 (6)**
Glucose (0.01) + lactate (10)	21 ± 1 (6)	65 ± 12 (6)
Glucose (1) + lactate (10)	1053 ± 219 (6)	942 ± 173 (6)
Glucose (5) + lactate (10)	1433 ± 294 (6)	1234 ± 99 (6)*
Glucose (0.01) + methyl pyruvate (10)	31 ± 1 (6)	23 ± 2 (6)*
Glucose (1) + methyl pyruvate (10)	366 ± 30 (6)**	338 ± 33 (6)*
Glucose (5) + methyl pyruvate (10)	438 ± 33 (6)**	310 ± 143 (4)**

Table 4 Effects of lactate, pyruvate and methyl pyruvate on rates of glucose oxidation in non-transfected INS-1 cells and in the LDH-overexpressing cell clone INS-LDH1

Cells were incubated for 60 min at 37 °C in the presence of various concentrations of D-[U-^{14}C]glucose, plus the added agent at a concentration of 10 mM. Glucose oxidation rates were calculated from production of $^{14}CO_2$. Values are means ± S.E.M. for the numbers of independent experiments given in parentheses. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control cells in the presence of the same concentration of glucose alone.

	Glucose oxidation (μmol/h per g of DNA)	
Additions to medium (mM)	INS-1	INS-LDH1
Glucose (0.01)	44 ± 8 (4)	43 ± 11 (4)
Glucose (1)	307 ± 11 (4)	268 ± 21 (4)
Glucose (5)	366 ± 4 (4)	290 ± 15 (4)
Glucose (0.01) + pyruvate (10)	9 ± 1 (5)**	10 ± 1 (5)*
Glucose (1) + pyruvate (10)	63 ± 11 (5)**	89 ± 9 (5)**
Glucose (5) + pyruvate (10)	79 ± 14 (5)**	152 ± 7 (5)**
Glucose (0.01) + lactate (10)	9 ± 1 (5)**	9 ± 1 (5)*
Glucose (1) + lactate (10)	103 ± 11 (5)**	107 ± 13 (5)**
Glucose (5) + lactate (10)	125 ± 9 (5)**	160 ± 14 (5)**
Glucose (0.01) + methyl pyruvate (10)	3 ± 0 (5)**	4 ± 0 (6)**
Glucose (1) + methyl pyruvate (10)	25 ± 3 (6)**	45 ± 2 (6)**
Glucose (5) + methyl pyruvate (10)	41 ± 4 (6)**	52 ± 2 (6)**

glucose-unresponsive RINm5F cells [10], INS-1 cells showed the characteristic concentration-dependent insulin-secretory responsiveness at millimolar glucose concentrations [25,36]. The rate of insulin secretion increased to the same extent in both control and LDH-transfected INS-1 cells, thus providing a feasible model in which to test the functional role of LDH in metabolic signalling. There was no significant effect of LDH overexpression on glucose-induced insulin secretion, confirming an earlier observation [14].

LDH-A overexpression did not significantly affect the overall rate of glycolytic flux, as estimated from the glucose utilization rate measured by conversion of D-[5-^{3}H]glucose into $^{3}H_2O$, in either INS-1 and RINm5F cells. In contrast, glucose oxidation, as determined from the conversion of D-[U-^{14}C]glucose into $^{14}CO_2$, was decreased by 20–30% after LDH-A overexpression in the INS-LDH1 cell clone (which showed a 25-fold increase in LDH activity), but not in the other INS-LDH clones with lower levels of LDH overexpression or in the RINm5F cell clones. This is an indication of increased metabolic flux in the direction of lactate. This interpretation is supported by the significantly higher rate of lactate production observed after LDH-A overexpression. On the other hand, the unchanged rate of glucose-stimulated insulin secretion even in the INS-LDH1 cell clone shows that the observed 20–30% decrease in the glucose oxidation rate after LDH-A overexpression does not apparently divert a critical portion of the signal generating metabolic flux from oxidative mitochondrial metabolism to anaerobic lactate production.

This inhibition of glucose oxidation occurred only at a high millimolar, but not at a lower millimolar or a micromolar, glucose concentration. Thus our observation indicates that the glucokinase component of glucose metabolism is more sensitive to the negative effects of LDH-A overexpression than the high-affinity hexokinase component. This observation implies a functional relationship between the glucokinase-mediated glycolytic flux and LDH-A in insulin-releasing cells.

In RINm5F cells, with a hexokinase-dominated glycolytic flux and in which the constitutive high level of LDH-A expression was increased only 2–3-fold through additional LDH-A overexpression, rates of both glucose utilization and glucose oxidation remained unaffected by LDH-A overexpression at all glucose concentrations. Insulin release from these RINm5F cells remained glucose-insensitive. Thus a further increase in the high constitutive level of expression of LDH-A did not overcome the glucose insensitivity of the RINm5F cells. All our data thus indicate that high levels of LDH favour the anaerobic conversion of pyruvate into lactate.

Oxidation of pyruvate and lactate is not affected by LDH-A overexpression in INS-1 cells [14]. As we show in the present study, pyruvate and lactate have significant effects upon insulin

secretion and glucose metabolism in LDH-overexpressing cells. In control INS-1 cells, pyruvate and lactate stimulated insulin secretion in the absence of glucose to a comparable extent, and potentiated glucose-induced insulin secretion at 1 and 5 mM glucose [12].

In the LDH-A-overexpressing INS-LDH1 cell clone (25-fold increase in LDH activity), the significant potentiating effects of both pyruvate and lactate on insulin secretion were lost. This loss of the effect of pyruvate on insulin secretion can be explained by two mechanisms. The first of these is increased flux through LDH-A, producing lactate; due to consumption of NADH from glycolysis, this removes some of the glycolytically generated reducing equivalents from ATP synthesis [37]. The observed decrease in glucose utilization in the presence of pyruvate supports the existence of this competition between LDH and the mitochondrial shuttles for re-oxidation of the cytosolic NADH [12]. As a result, insulin secretion would be decreased due to a decrease in ATP generation [37]. In addition, overexpression of LDH-A in glucose-responsive insulin-secreting cells, by increasing the rate of conversion of pyruvate into lactate, is likely to decrease the amount of exogenously administered pyruvate that is channelled into a signal generating mitochondrial metabolism [12].

The inhibitory effect of lactate on glucose-induced insulin secretion in INS-1 cells overexpressing LDH-A could be explained through a mass-action effect, i.e. reversal of the reaction catalysed by LDH due to the high lactate concentration [14]. This might increase the cytosolic redox state more efficiently in cells overexpressing LDH, resulting in a partial reversal of the equilibrium catalysed by glyceraldehyde phosphate dehydrogenase towards glyceraldehyde 3-phosphate and a subsequent decrease in the glycolytic flux, finally resulting in a decrease in ATP synthesis [38].

Because of the lipophilic character of the ester, the cellular uptake of methyl pyruvate is not dependent upon the expression of a monocarboxylate transporter [39]. The insulin-secretory response induced by methyl pyruvate was similar to that due to pyruvate, suggesting that uptake of pyruvate into INS-1 cells is not severely limited by the level of expression of the monocarboxylate transporter in these cells. This is in accordance with the observation of Ishihara et al. [14], who found no increase in pyruvate-stimulated insulin secretion after overexpression of the monocarboxylate transporter in INS-1 cells. However, the insulin-secretory potency of methyl pyruvate was diminished in LDH-A-overexpressing INS-1 cells, with the increased secretory effect being completely lost at 5 mM glucose. As in the case of exogenously added pyruvate, this loss of insulin-secretory potency can be interpreted as being the result of decreased ATP generation due to loss of NADH in the LDH reaction [12].

Thus the results of the present study provide evidence that the level of expression of LDH in glucose-responsive insulin-secreting cells is of critical importance for the correct partitioning of aerobic and anaerobic glucose metabolism with respect to its signal function for hexose-induced insulin secretion [40–42]. Interestingly, it was the glucokinase-mediated glycolytic flux in particular that was decreased in INS-1 cells by LDH-A overexpression. Thus the preferential channelling of glucose to aerobic metabolism by glucokinase [43] in INS-1 cells may be determined, at least in part, by the low level of constitutive expression of LDH-A in these insulin-secreting cells [12], thus securing the unique role of glucose as the principal physiological stimulus of insulin secretion in rodents and in humans [5,44,45].

A complete absence of LDH activity in pancreatic β-cells would without doubt protect the organism from an undesirable insulin-secretory effect of lactate. However, this protection would have to be 'paid for' by an unwanted insulin-secretory effect of exogenous pyruvate.

A very high level of expression of LDH, on the other hand, would suppress undesirable insulin-secretory effects of both exogenous pyruvate and lactate. However, this could endanger correct glucose-induced insulin secretion by diverting too much signal generating glycolytic flux from being channelled into aerobic metabolism, as it may overcharge the capacity of the glucokinase-mediated signal generating metabolic flux [5].

Thus restricted expression of LDH [12], in combination with a low level of expression of the monocarboxylate transporter in the plasma membrane [14,46] and sufficient activity of mitochondrial glycerol phosphate dehydrogenase [12,13] represents a compromise for the pancreatic β-cell. It ensures that pyruvate and lactate do not act as insulin secretagogues [7,15–20]. At the same time, it secures glucose responsiveness of insulin secretion and prevents loss of the responsiveness of insulin secretion to glucose stimulation in situations of a decrease in the glucokinase-mediated signal generating metabolic flux, i.e. during a re-feeding phase after periods of starvation, which decrease glucokinase activity in pancreatic β-cells [5,47].

The skilful technical assistance of D. Lischke and A. Petzold is gratefully acknowledged. O. A. was recipient of a Marie-Curie Grant in the TMR programme of the European Union.

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Received 18 May 2000/11 August 2000; accepted 15 September 2000