Acute Overexpression of Lactate Dehydrogenase-A Perturbs β-Cell Mitochondrial Metabolism and Insulin Secretion

Edward K. Ainscow, Chao Zhao, and Guy A. Rutter

Islet β-cells express low levels of lactate dehydrogenase and have high glycerol phosphate dehydrogenase activity. To determine whether this configuration favors oxidative glucose metabolism via mitochondria in the β-cell and is important for β-cell metabolic signal transduction, we have determined the effects on glucose metabolism and insulin secretion of acute overexpression of the skeletal muscle isoform of lactate dehydrogenase (LDH)-A. Monitored in single MIN6 β-cells, LDH hyperexpression (achieved by intranuclear cDNA microinjection or adenoviral infection) diminished the response to glucose of both phases of increases in mitochondrial NAD(P)H, as well as increases in mitochondrial membrane potential, cytosolic free ATP, and cystolic free Ca^{2+}. These effects were observed at all glucose concentrations, but were most pronounced at submaximal glucose levels. Correspondingly, adenoviral vector-mediated LDH-A overexpression reduced insulin secretion stimulated by 11 mmol/l glucose and the subsequent response to stimulation with 30 mmol/l glucose, but it was without significant effect when the concentration of glucose was raised acutely from 3 to 30 mmol/l. Thus, overexpression of LDH activity interferes with normal glucose metabolism and insulin secretion in the islet β-cell type, and it may therefore be directly responsible for insulin secretory defects in some forms of type 2 diabetes. The results also reinforce the view that glucose-derived pyruvate metabolism in the mitochondrion is critical for glucose-stimulated insulin secretion in the β-cell. *Diabetes* 49:1149–1155, 2000

Stimulation of insulin secretion by glucose is likely to involve the metabolism of the sugar through glycolysis and the subsequent oxidation of pyruvate and NADH by mitochondria (1). This activation of oxidative metabolism may then prompt an increase in the free concentrations of intracellular ATP and MgATP (2,3), causing the closure of ATP-sensitive K^+ channels (4) and leading to Ca^{2+} influx and the activation of exocytosis.

Mitochondrial-linked metabolism accounts for >80% of glucose metabolism in the β-cell (5,6), a much greater proportion than that seen in most other mammalian cell types. This observation is consistent with the idea that activated mitochondrial metabolism, which furnishes dramatically more ATP per glucose molecule than glycolysis alone, may be the trigger for insulin secretion in response to elevated glucose concentrations (1). However, it is also conceivable that vigorous oxidative metabolism is necessary principally to supply the considerable energetic demands of insulin biosynthesis, whereas the key signaling molecule(s) responsible for triggering K_{ATP} channel closure and exocytosis of insulin could be derived from glycolysis (7). A means of altering the relative contributions of glycolysis and mitochondrial oxidation to overall glucose utilization would allow these 2 hypotheses to be tested.

It is unclear what mechanisms achieve the preferential oxidation of glucose-carbon atoms by mitochondria. We have previously proposed that the remarkably low levels of lactate dehydrogenase (LDH) expressed by primary islet β-cells and well-differentiated β-cell lines (5,8–10)—as well as low plasma membrane lactate transport activities (5) and high levels of mitochondrial glycerol phosphate dehydrogenase activity (5,11)—may well be important. Thus, the absence of significant LDH activity in β-cells could be important to ensure that a high proportion of both pyruvate and NADH, furnished by glycolysis, is subsequently oxidized by mitochondria. Indeed, elevated LDH levels observed in stably transfected MIN6 cells (9) and in the islets of rodents rendered diabetic by 95% pancreatectomy (10) are associated with impaired glucose-stimulated insulin secretion. However, the expression of other genes is also altered in the islets of pancreatectomized animals (10) and possibly in stably transformed MIN6 cells. These changes could contribute to the observed decrease in glucose responsiveness in each of these models.

In the present study, we have sought to examine in detail the effects of more acute overexpression of cDNA encoding LDH-A (the skeletal muscle LDH isoform), achieved either through direct intranuclear microinjection of plasmids into single MIN6 β-cells or through infection of cell populations with a recombinant adenoviral vector. Our principal aims have been to determine the effects of these maneuvers on *1*) glucose-stimulated secretion and *2*) intracellular metabolism of and signaling by the sugar, as gauged dynamically at the single-cell level through changes in reduced mitochondrial pyri-

From the Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol, U.K.

Address correspondence and reprint requests to Guy A. Rutter, PhD, Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD, U.K. E-mail: g.a.rutter@bris.ac.uk.

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$[ATP]_c$ cytosolic free ATP concentration; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial free $[Ca^{2+}]$; CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; KRBH, Krebs-Ringer bicarbonate HEPES; LDH, lactate dehydrogenase; LDH-A+, cells overexpressing LDH-A; NAD(P)H, reduced mitochondrial pyridine nucleotides; RT-PCR, reverse transcriptase–polymerase chain reaction; TMREE, tetramethyl rhodamine ethyl ester; $\Delta\Psi$, mitochondrial membrane potential.

dine nucleotides [NAD(P)H], mitochondrial membrane potential ($\Delta\Psi$), cytosolic free ATP concentration ($[ATP]_c$), and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$). The use of single-cell imaging has allowed us *1*) to exploit intranuclear cDNA microinjection, which permits the introduction of a large number of plasmid molecules and thus highly efficient protein expression; *2*) to compare the responses of adjacent control and LDH-A overexpressing cells, cultured and stimulated simultaneously on the same coverslip; and *3*) to avoid the potential complication that the responses observed in a cell population actually reflect a progressive recruitment of individual cells (12,13).

Using these approaches, we demonstrate that forced overexpression of LDH-A markedly inhibits intracellular metabolism and signaling by glucose, as well as stimulated insulin secretion, at submaximal concentrations of the nutrient. Furthermore, when MIN6 cells are first preincubated at intermediate glucose concentrations, subsequent increases in glucose to supramaximal levels (30 mmol/l) stimulate secretion much more weakly in cells overexpressing LDH-A. In contrast, LDH-A overexpression does not affect the increase in $[Ca^{2+}]_c$ or insulin secretion after a single-step increase in glucose from substimulatory (3 mmol/l) to supramaximal levels (30 mmol/l), even though clear effects of LDH-A expression on mitochondrial glucose metabolism are still evident.

Some of these results have previously been reported in abstract form (14).

RESEARCH DESIGN AND METHODS

Cell culture, microinjection, and imaging. MIN6 β-cells (15) were cultured essentially as described in (2) and were used between passage #25 and #32. Briefly, cells were routinely cultured in Dulbecco's modified Eagle's medium containing 25 mmol/l glucose. For experimental use, cells were grown on poly-L-lysine–treated glass coverslips and incubated overnight in medium containing 3 mmol/l glucose before the measurements of glucose responses.

Plasmids (0.2–0.4 mg/ml) were microinjected 24 h before imaging, as previously described (16–18). For fluorescence imaging, a plasmid containing LDH-A cDNA (19) under the control of a cytomegalovirus (CMV) promoter from pcDNA1 (Invitrogen, Groningen, the Netherlands) was coinjected into cells simultaneously with pEGFP-N1 (Clontech, Basingstoke, U.K.). The latter plasmid acted as a marker for those cells that had been successfully microinjected. In control experiments, pEGFP-N1 alone was injected into the cells.

Autofluorescence due to NAD(P)H was measured using a Leica TCS-NT (Leica, Heidelberg, Germany) inverted confocal system fitted with a ×40 oil immersion objective. An ultraviolet laser (Coherent, Auburn, CA), with spectral lines at 351 nm and 364 nm, was used for excitation, and emission at 440 nm was measured and analyzed using Leica TCS software. Microinjected cells were visualized via expressed enhanced green fluorescent protein (EGFP) fluorescence using a Krypton/Argon laser with excitation wavelengths of 488 nm and emission at 530 nm. During imaging, cells were maintained on a temperature-controlled (37°C) stage in modified Krebs-Ringer bicarbonate HEPES (KRBH) medium comprising 125 mmol/l NaCl, 3.5 mmol/l KCl, 1.5 mmol/l $CaCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2PO_4, 2.5 mmol/l $NaHCO_3$, 10 mmol/l HEPES-Na^+, pH 7.4, which initially contained 3 mmol/l glucose and equilibrated with 95:5 O_2:CO_2. Changes in glucose concentration were made by the addition of a small volume of stock solution of 1.5 mol/l glucose followed by mixing of the solution with a pipette. In any given experiment, 1–5 cells in the field could be identified as expressing EGFP, and the responses of NAD(P)H fluorescence in these cells to changes in glucose concentration in the medium were compared with 5 neighboring (control) cells. The fluorescence signal was normalized to the average signal at 3 mmol/l glucose.

Changes in $\Delta\Psi$ were measured using tetramethyl rhodamine ethyl ester (TMREE) fluorescence, as described previously (20), on the same system as that used for NAD(P)H measurements, using a Krypton/Argon laser with excitation wavelength 568 nm and emission filter at 590 nm.

Changes in $[Ca^{2+}]_c$ in response to glucose were measured by following changes in fura-2 fluorescence. Cells were loaded for 40 min in KRBH containing 3 mmol/l glucose, 5 μmol/l fura-2-AM (Sigma, Poole, Dorset, U.K.) and 0.05% Pluronic F-127 (BASF, Mount Olive, NJ). Before imaging, cells were washed twice and then incubated in KRBH in the absence of fura-2-AM. Imaging was performed on a Leica DM/IRBE inverted epifluorescence microscope fitted with a × 40 oil immersion objective and 450 nm long pass dichroic mirror. The emission at 510 nm during alternate excitation (LEP Biopoint wheel; LudL Electronic Products, Munich, Germany) at 340 nm and 380 nm (Omega Optical filters, Brattleboro, VT) was detected with a Hamamatsu C4742–995 charge-coupled device camera (Hamamatsu, Hamamatsu City, Japan). The 340/380 ratio was calculated off-line using Openlab (Warwick Science Park, U.K.) software and used as a measure of $[Ca^{2+}]_c$. Changes in glucose concentration were achieved as above. EGFP expression (detected by fluorescence excitation at 488 nm) was again used a marker for successful microinjection. Because there was a degree of fluorescence detected in EGFP-expressing cells not loaded with fura-2, when excited at 380 nm (~20% of the fluorescence at 380 nm derived from fura-2), direct calibration of the fura-2 signal in terms of absolute $[Ca^{2+}]_c$ was not possible. Instead, at the end of each experiment, cells were exposed to 70 mmol/l KCl, which causes a large (>4 μmol/l) and rapid increase in $[Ca^{2+}]_c$ (21). The fluorescence ratio changes observed upon changes in glucose concentration in the medium were then expressed as a percentage of the difference between basal (i.e., in the presence of 3 mmol/l glucose; R_{min}) and maximal $[Ca^{2+}]_c$ (i.e., in the presence of 70 mmol/l KCl; R_{max}). To verify the validity of this normalization, we performed a simulation of the effect of EGFP expression on the observed changes in 340/380 ratio. When the ratio change (R) is considered as a fraction of the total range of ratio change—i.e., $(R - R_{min})/(R_{max} - R_{min})$—then the discrepancy between the observed change in 340/380 fluorescence and that derived from fura-2 changes is negligible (an overestimate of no greater than 2%).

For $[ATP]_c$ measurements (22), using luciferase luminescence imaging (2), cells were coinjected with a plasmid containing cDNA encoding humanized firefly-luciferase from plasmid pGL3 (Promega, Southampton, U.K.) under the CMV promoter of plasmid pcDNA 3 (Invitrogen). Cells (~200) were microinjected within a defined area (~1 mm^2) on the coverslip, which was divided into 2 adjacent regions. In 1, cells were coinjected with pcDNA3.LDH-A and in the other, empty pcDNA3 alone. After a 24-h culture, light output from cells in each area was imaged simultaneously and in real time, as previously described (2).

Adenovirus generation and insulin secretion. Recombinant adenovirus containing human LDH-A cDNA (AdLDH.EGFP) and a control adenovirus (AdEmpty.EGFP) were prepared using AdEasy system (23). The adenoviral and shuttle vectors were provided by Dr. F Gunn-Moore, University of Edinburgh. Briefly, a 1.7 kb *Kpn*1/*Xba*1 fragment comprising full-length human LDH-A was restricted from plasmid LDHA.pcDNA3 and ligated into a shuttle vector, pAdTrack.CMV, under the control of a CMV promoter. The generated construct contained cDNA encoding both LDH-A and EGFP, each under the control of a separate CMV promoter. Cotransformation and recombination of the above construct and adenoviral vector pAdEasy-1 resulted in a recombinant adenoviral plasmid. The adenoviral plasmid was then transfected into HEK 293 cells, and adenovirus particles were obtained by extracting the cells 7–10 days after transfection. EGFP expression was used to monitor the production of the virus. The virus was amplified and CsCl gradient was centrifugation-purified. Stocks of 10^9–10^{10}/ml infectious particles were retained and used for experiments. Titration of the virus was performed by infecting HEK 293 cells with serially diluted viral stocks and counting positive cells through EGFP fluorescence (488 nm excitation).

To infect MIN6 cells, recombinant LDH-A–containing or control adenovirus solution was mixed with culture medium, and cells were exposed to the virus with 100 multiplicity of infection for 1 h. Cells were then washed, and the medium was replaced. Cells were used for experiments 24–48 h after infection. Under these conditions, >85% of cells were infected, as determined by EGFP expression.

Reverse transcriptase–polymerase chain reaction (RT-PCR) from the total RNA of infected MIN6 cells was used to confirm expression of LDH-A from the virus (Fig. 1*A*). RT-PCR was performed in 2 steps: RT of 100 ng total RNA was performed with the reverse primer using Tth DNA polymerase (Roche Molecular Biologicals, Lewes, East Sussex, U.K.); and then the RT product underwent a 30-cycle PCR amplification using the Expand Hi-Fidelity PCR system (Roche Molecular Biologicals). The following primers were used: forward 5′-TTGTGAAGGTGACTCTGACT, reverse 5′-GTTTTAGTTGGTATAACACTTG. Direct confirmation of LDH-A overexpression was obtained from assay of LDH activity (9) in infected and control (null-virus–infected) cells.

Insulin secretion was measured from monolayer culture MIN6 cells (~300,000) grown on glass coverslips and infected with either AdLDH.EGFP or Adempty.EGFP before use. Insulin secretion was followed dynamically using a temperature-controlled perifusion chamber (built in-house; volume ~100 μl). The chamber was perifused (1 ml/min) at 37°C with KRBH medium supplemented with 0.1% bovine serum albumin and initially 3 mmol/l glucose. After a preincubation of 15 min, fractions of the perifusate were collected at 1-min intervals. Subsequently, the glucose concentration in the perifusion medium was changed as indicated (Fig. 1*B* and *C*). Released insulin was mea-

sured using a radioimmunoassay kit (No. RI-13K, Linco, St. Charles, MO). Responses to glucose are expressed relative to basal secretion. Extraction of insulin from whole cells was performed as described (24).

Statistical analysis. Unless stated otherwise, significance tests were performed using a paired 1-tailed Student's *t* test. Nonlinear regression was performed by minimizing the sum of squares of the residuals to the fitted curves using the solver function within Microsoft Excel v.5.0.

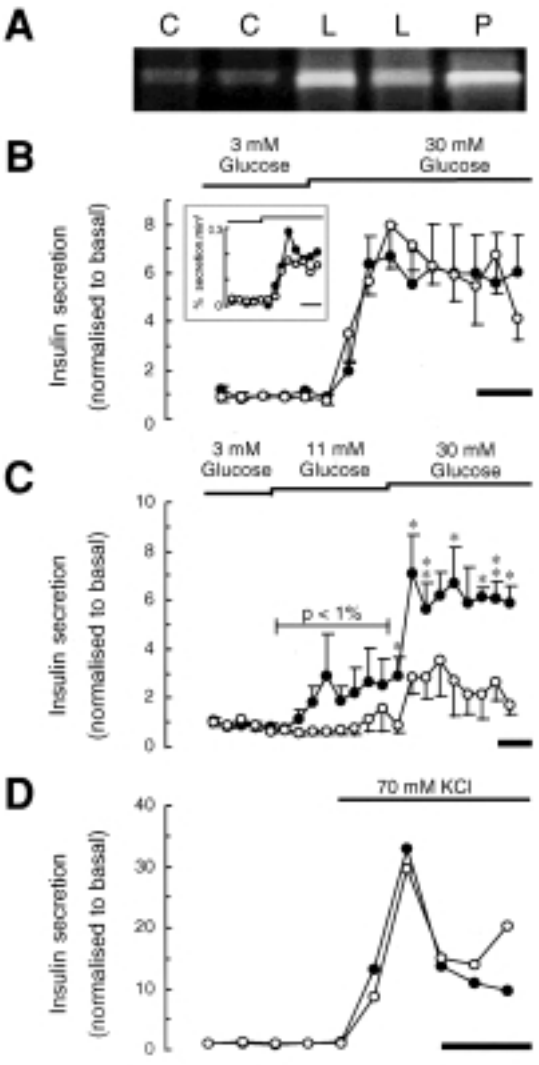

FIG. 1. Effect of LDH-A expression on glucose-stimulated insulin secretion. ***A*****: RT-PCR of mRNA from MIN6 cells infected with AdEmpty.EGFP (C) and AdLDH.EGFP (L) demonstrated that AdLDH.EGFP caused overexpression of LDH-A. The final lane (P) shows results of PCR of isolated LDH-A cDNA confirming the specificity of the probes used.** ***B:*** **Cells were infected with either AdEmpty.EGFP (●, error bars are plus SE) or AdLDH.EGFP (○, error bars are minus SE) before measurement of insulin secretion in a perifusion chamber (see RESEARCH DESIGN AND METHODS). The fold increase in insulin secretion over basal, i.e., in the presence of 3 mmol/l glucose, is presented. Data are means of 4 experiments where glucose was stepped directly to 30 mmol/l. Similar responses were observed upon normalization to the total extractable insulin—shown in the inset, data from a single experiment.** ***C:*** **Data are the means of 3 experiments where glucose was stepped to 11 and then 30 mmol/l. Null-virus–infected cells displayed a significantly higher rate of secretion ($P < 1\%$) within each experiment when time points during incubation at 11 mmol/l glucose were compared.** ***D:*** **The secretory responses of either AdEmpty.EGFP or AdLDH.EGFP infected cells to 70 mmol/l KCl are shown. Data are means of 2 separate experiments. The time bar indicates 200 s.**

RESULTS

Effect of LDH overexpression on glucose-induced insulin secretion and $[Ca^{2+}]_c$ increases. Infection of MIN6 cells with recombinant adenovirus encoding LDH-A cDNA led to a clear increase in the level of LDH mRNA (Fig. 1*A*) and a 2.6-fold increase in total LDH activity (μmol · min^{-1} · mg · $protein^{-1}$), from 0.049 ± 0.021 (null virus) to 0.15 ± 0.065 (LDH-A virus; n = 3 separate experiments). Western blotting using polyclonal antiserum to LDH-A (Biogenesis, Poole, Dorset, U.K.) confirmed the overexpression of LDH-A protein upon infection with the LDH-A–containing adenovirus (data not shown).

As shown in Fig. 1*B*, adenovirus-mediated LDH-A expression had no significant effect on insulin secretion in perifused MIN6 β-cell populations stimulated by a single-step increase in glucose concentration from 3 to 30 mmol/l. Maximal stimulation by glucose gave a 6.4 ± 1.1–fold increase (n = 4 separate experiments) over basal secretion in LDH overexpressing cells and a 6.1 ± 1.4–fold increase (n = 4) in control infected cells (Fig. 1*B*). Basal secretion in AdEmpty.EGFP infected cells was 0.033 ± 0.012% of total insulin/min (n = 5), whereas the corresponding figure for cells infected with AdLDH.EGFP was 0.039 ± 0.012% of total insulin/min (n = 5) (Fig. 1*B*, inset).

By contrast, prior perifusion for 8 min at suprathreshold but submaximal glucose concentrations (11 mmol/l) resulted in a markedly poorer stimulation of insulin secretion and dramatically reduced the response to a further increase in glucose concentration to 30 mmol/l (Fig. 1*C*). In control cells, insulin secretion was 6.2 ± 1.5 fold (n = 3) above basal, similar to cells that were not pre-exposed to 11 mmol/l glucose. However, in cells overexpressing LDH-A (LDH-A+), stimulation with 30 mmol/l glucose following 11 mmol/l glucose caused insulin secretion at only 2.6 ± 0.8 fold (n = 3, $P < 5\%$ compared with null-infected) above the basal rate. In contrast, secretion stimulated with 70 mmol/l KCl was not significantly different in control and LDH-A+ cells (Fig. 1*D*).

These effects of LDH-A overexpression were closely paralleled at the single-cell level in the responses to glucose of changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$). In these experiments, adjacent single cells were microinjected with a mixture of cDNAs encoding EGFP and LDH-A, were microinjected with EGFP-expressing plasmid alone, or were left uninjected. After expression of the constructs, $[Ca^{2+}]_c$ changes in response to glucose were monitored after loading with the trappable fluorescent indicator, fura-2. Importantly, this approach allowed comparison of the responses in adjacent (LDH-A+ and control) cells imaged simultaneously within the same field. However, a drawback of this method was the fact that EGFP weakly absorbed excitatory light at 380 nm. Therefore, direct comparison of the changes in the fluorescence ratio at 340 nm to 380 nm by fura-2 between adjacent cells was not possible. This was overcome by expressing the ratio changes as a percentage of the increase observed over basal when $[Ca^{2+}]_c$ was maximally elevated (~4 μmol/l) (17) by the addition of 70 mmol/l KCl (see RESEARCH DESIGN AND METHODS).

As observed for insulin secretion, coexpression of LDH-A (and EGFP) had no effect on the response of $[Ca^{2+}]_c$ to glucose in MIN6 cells stimulated with a single-step change from 3 to 30 mmol/l glucose (Fig. 2*A*). However, a diminished response to glucose was observed when cells were incubated in 11 mmol/l and subsequently 30 mmol/l glucose (Fig. 2*C*). Expression of EGFP alone had no significant effect on the

response to 11 followed by 30 mmol/l glucose after normalization of the 340/380 nm fluorescence ratio (Fig. 2*B*, *inset*).

Effects of LDH-A overexpression on glucose-stimulated oxidative metabolism. Further investigation of the origin of the different responses observed in LDH-A+ and control cells focused on the response of mitochondrial metabolism to changes in glucose concentration. The position of LDH in glucose metabolism is such that it forms a branch point, diverting pyruvate and NADH away from mitochondrial oxidation. Hence, it is possible that overexpression of LDH-A may lead to altered mitochondrial metabolism. In this study, we monitored 3 key intermediates in the glucose signaling pathway: NAD(P)H (autofluorescence at 340 nm) (25–27), $\Delta\Psi$ (measured with the dye TMREE) (2,28), and $[ATP]_c$ concentration (detected as changes in the luminescence of expressed firefly luciferase) (2).

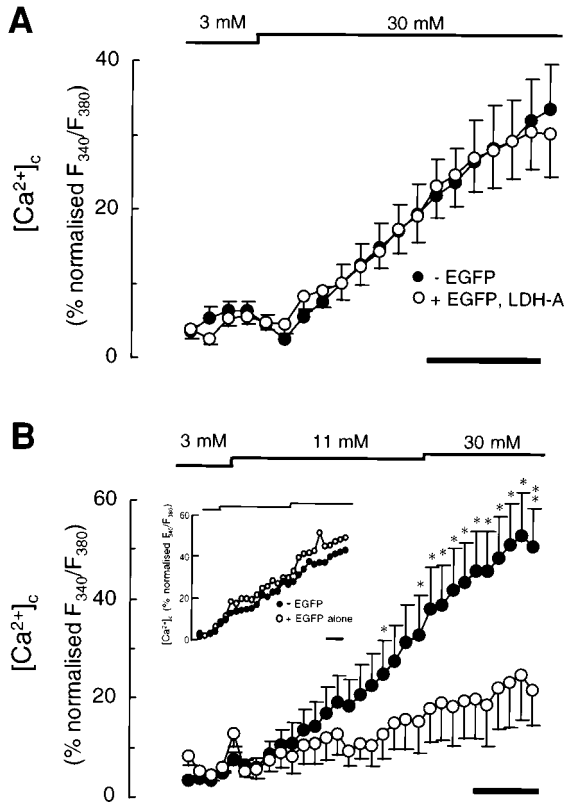

FIG. 2. Effect of LDH-A expression on the response of $[Ca^{2+}]_c$ to glucose. Cytoplasmic $[Ca^{2+}]$ changes in cells coinjected with EGFP and LDH-A cDNAs (○, error bars are minus SE) and uninjected adjacent cells (●, error bars are plus SE) were monitored by changes in F_{340}/F_{380} of entrapped fura-2. Fluorescence ratio changes were expressed as a percentage of the maximum change in ratio achieved by addition of 70 mmol/l KCl to the medium (see RESEARCH DESIGN AND METHODS). In *A*, LDH-A+ cells (n = 16) (co-microinjected with plasmids pEGFP.N1 and pcDNA1.LDH-A) were compared with cells (n = 22) from 4 cultures upon a single-step change in glucose from 3 to 30 mmol/l glucose. In *B*, LDH-A+ cells (n = 12) were compared with cells (n = 20) from 4 cultures upon a 2-step change in glucose from 3 to 11 to 30 mmol/l. The average responses of cells (n = 12) injected with pEGFP-N1 alone and uninjected cells (n = 16) from 3 cultures to this 2-step change in glucose are shown in the inset (error bars omitted for clarity). Note that the EGFP fluorescence did not interfere with the estimate of fura-2 F_{340}/F_{380} ratio changes, after the normalization procedure. The time bar represents 200 s.

Cellular ultraviolet autofluorescence (340 nm excitation, 440 nm emission) is primarily due to NADH and NADPH [collectively termed NAD(P)H] within mitochondria (29,30). Microinjection of EGFP and LDH-A cDNA-containing plasmids was again used to compare responses to glucose of adjacent cells in a single monolayer culture. The principle of the method is shown in Fig. 3*A* and *B*. Examined in optical sections by laser-scanning confocal microscopy, autofluorescence was apparent purely in the extranuclear compartment, indicating that only mitochondrial, and not cytosolic, NAD(P)H (which equilibrates into the nucleus) was being measured, as expected.

In response to a single-step change from 3 to 30 mmol/l glucose, control cells injected with EGFP cDNA alone gave identical responses to adjacent, noninjected cells (Fig. 3*A*), whereas those coinjected with LDH-A cDNA displayed an altered response (Fig. 3*B* and *C*). Essentially identical results were obtained with parallel cultures infected with the AdLDH.EGFP and AdEmpty.EGFP viruses (not shown). This is in contrast to the absence of effects of LDH overexpression on glucose-stimulated insulin secretion and $[Ca^{2+}]_c$.

Examination of the response of NAD(P)H fluorescence to increases in extracellular glucose typically showed a biphasic pattern that could be fitted to 2 exponential curves by nonlinear regression (Fig. 3*C*). This biphasic response was seen in 23 of 26 separate experiments (each the mean of 5 individual cells). Previous studies have shown similar responses in single primary β-cells (25) and islets (26,27). The first phase (phase I) resulted in a rapid increase in fluorescence of 26 ± 4% (amplitude of fitted exponential, n = 5 separate cell cultures, 25 cells). The second, slower, phase (phase II) began 280 ± 20 s (n = 5 cultures) after addition of 30 mmol/l glucose and caused an increase of 37 ± 11% (amplitude of fitted exponential, n = 5 cultures). The amplitude of both phases of NAD(P)H response was diminished in cells expressing LDH-A (Fig. 3*D*), with the second phase showing the greater difference, being reduced by close to 80%. A significant difference was also observed in the time of onset of the second phase, which was 390 ± 30 s (n = 5 cultures, 14 cells) in LDH-A+ cells.

In common with the changes in NAD(P)H, the initial response of $\Delta\Psi$ to a change in extracellular glucose concentration from 3 to 30 mmol/l was barely affected in LDH-A+ cells. No difference in the response of $\Delta\Psi$ to 30 mmol/l glucose was observed in cells co-microinjected with plasmid pEGFP.N1 alone (Fig. 3*E* and *F*). However, at times >250 s after glucose stimulation, there were significant differences in the observed TMREE fluorescence (Fig. 3*G* and *H*), with the response being impaired in LDH-A+ cells.

In contrast, changes in $[ATP]_c$—monitored via following light output from cells injected with firefly luciferase cDNA—were significantly altered after overexpression of LDH-A immediately following an increase in glucose concentration from 3 to 30 mmol/l and also at >250 s (Fig. 3*I* and *J*). The initial drop in $[ATP]_c$ is presumably due to phosphorylation of glucose (and hence consumption of ATP) by glucokinase. The rate of this process appeared to be more pronounced in LDH-A+ cells, possibly reflecting enhanced glycolytic flux. However, the lower $[ATP]_c$ observed in LDH-A+ cells compared with control cells at later time points may reflect altered yield of ATP from oxidative phosphorylation, consistent with the observed differences in mitochondrial NAD(P)H (Fig. 3*B–D*) and $\Delta\Psi$ (Fig. 3*G* and *H*).

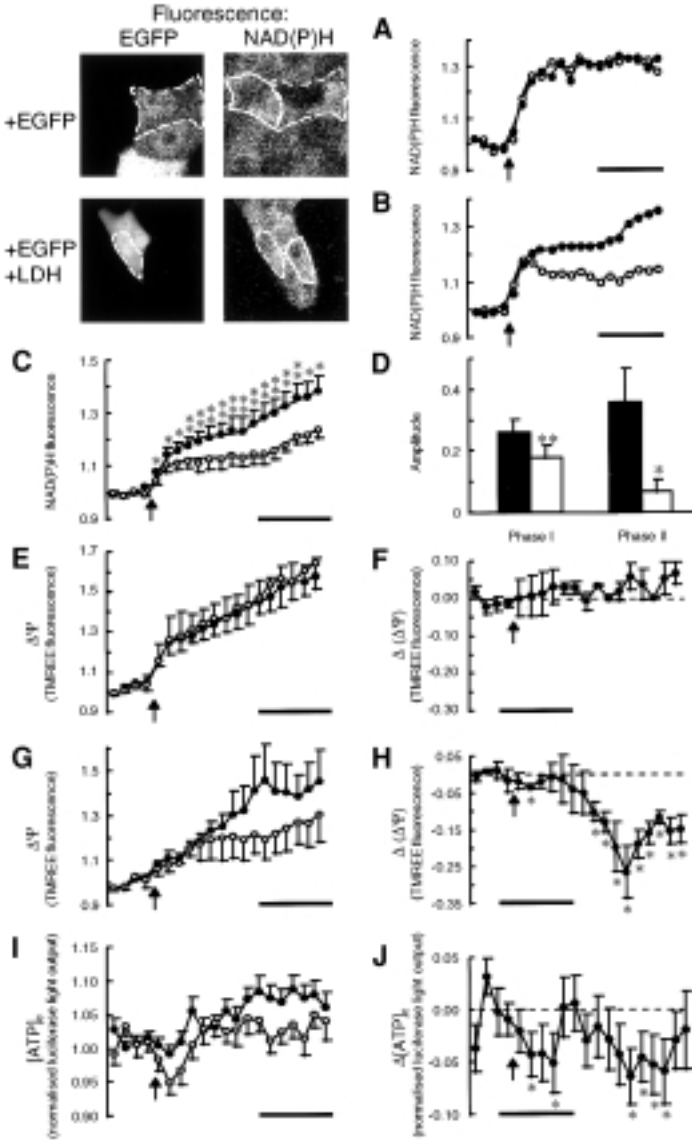

FIG. 3. Effect of LDH-A expression on metabolism during stimulation by 30 mmol/l glucose. *A:* Response of NAD(P)H to a stepped change from 3 to 30 mmol/l glucose (upward arrow) of an uninjected single MIN6 cell (●) or a cell microinjected plasmid pEGFP-N1 alone (○). *B:* As in *A* with uninjected cell (●) or cell injected with plasmid pcDNA3.LDH-A and pEGFP-N1 (○). *C:* The mean response (± SE) normalized to prestimulatory fluorescence, is shown for 25 uninjected and 14 LDH-A+ single cells from 5 separate cultures, treated as in *B*. *D:* A double exponential curve was fitted to both sets of data in *C* to obtain the amplitude of the 2 exponentials. *E*: Response of $\Delta\Psi$, measured in uninjected (●) and injected (pEGFP-N1 alone) (○) cells. *F*: Difference in glucose-induced change in $\Delta\Psi$ observed between EGFP expressing and control cells (mean ± range of 2 cultures, 12 cells each), derived from *E*. *G* and *H:* As in *E* and *F* but comparing uninjected cells (●) and cells coinjected with pEGFP-N1 and pcDNA3.LDH-A (○) (mean ± SE of 3 cultures, 16 and 11 cells, respectively). *I:* Measurements of $[ATP]_c$ using luminescence of cells injected with cDNA encoding firefly luciferase. Light output was integrated over 30 s. The average response of 27 LDH-A+ cells (coinjected with pcDNA3.luciferase and pcDNA3.LDH-A; ○) and 32 control cells (injected with pcDNA3.luciferase and empty pcDNA3; ●) from 4 separate experiments are shown, normalized to basal light output. Significances on the difference plots *D*, *H*, and *J* were tested using an unpaired 1-tailed Student's *t* test. The time bar represents 200 s in each case.

Under conditions in which a difference in insulin secretion and $[Ca^{2+}]_c$ was apparent, i.e., a stepped increase from 3 to 11 then to 30 mmol/l glucose, LDH-A+ cells displayed a substantial difference in responses of mitochondrial NAD(P)H fluorescence and $[ATP]_c$ compared with control cells imaged simultaneously (Fig. 4). For each parameter examined, prior exposure to a suprathreshold (but submaximal) glucose concentration resulted in a diminished response upon further elevation to 30 mmol/l glucose.

Demonstrating that the effects of LDH-A overexpression were specific for glucose-induced changes in metabolism, stimulation of mitochondrial metabolism achieved through an increase in intracellular $[Ca^{2+}]_c$ (and thus mitochondrial $[Ca^{2+}]$ $[Ca^{2+}{}_m]$) (31), provoked with external K^+ (32), was unaffected in LDH-A+ cells (Fig. 5*A*). Further, the effects of LDH-A to suppress mitochondrial metabolism were apparently not due to the blockade of Ca^{2+} uptake into mitochondria and thus a failure to stimulate mitochondrial dehydrogenases. Thus, the extent of inhibition of NAD(P)H increases was unaffected by the removal of external Ca^{2+} with EGTA (Fig. 5*B*). Under these conditions, increases in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in response to high glucose are completely blocked (32).

DISCUSSION

We explore here, in detail, the effects of forced and acute overexpression of LDH-A activity on glucose-stimulated metabolism and insulin secretion. We have used cells of the differentiated mouse MIN6 β-cell line, which display a robust, glucose-induced insulin secretion similar to islets (9,15). However, these cells are considerably more amenable to genetic manipulation (e.g., though cDNA microinjection) than islets or primary β-cells and can readily be cultured as a monolayer without loss of responsiveness to glucose. These features have allowed us to perform a careful analysis of mitochondrial metabolism through single-cell imaging techniques, enabling differences in cells to be observed in a single monolayer culture. Parallel studies using an adenoviral vector have allowed the

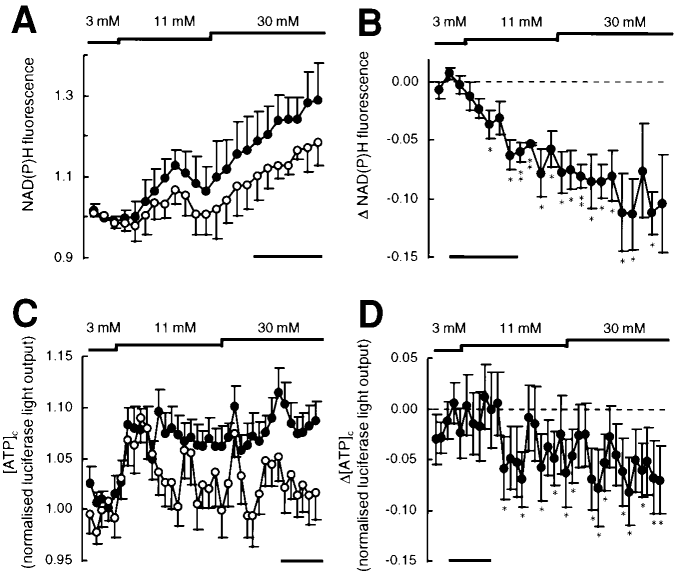

FIG. 4. Effect of LDH-A expression on metabolism during stimulation by 11 and then 30 mmol/l glucose. *A* and *B*: NAD(P)H data as a mean of 3 experiments with LDH-A+ cells (○) and control adjacent cells (●). *C:* $[ATP]_c$ changes as the mean from 25 LDH-A+ cells (○) and 36 control cells (●) from 4 experiments. Significances on the difference plot (*D*) were tested using an unpaired 1-tailed Student's *t* test. The time bar represents 200 s.

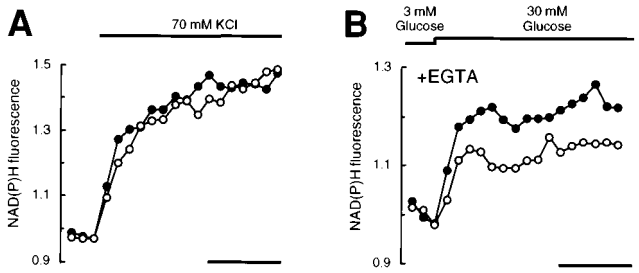

Fig. 5. Role of $[Ca^{2+}]_c$ in the LDH-A modulation of NAD(P)H metabolism. *A*: Response of NAD(P)H to 70 mmol/l KCl in LDH-A+ cells (*n* = 2 cells) and control cells (*n* = 4 cells). Each panel shows the results of a single experiment. *B*: The response of NAD(P)H to a change in glucose from 3 to 30 mmol/l glucose in LDH+ cells (○, *n* = 4 cells) and control cells (●, *n* = 4 cells) during incubation in EGTA-containing medium. See Fig. 3*C* for the response in the control (+Ca^{2+}) condition. The time bar represents 200 s.

effect on insulin secretion from a population of cells to be studied. In particular, it has been possible to achieve large increases in LDH-A activity at relatively short times after cDNA introduction, thus reducing the risk of nonspecific alterations in the expression of other genes whose products may also be involved in glucose-sensing.

Our first observation, in common with recent reports in islets (33) and stably transfected INS-1 cells (34), is that insulin secretion stimulated by a single stepped increase in glucose concentration from substimulatory (3 mmol/l) to supramaximal (30 mmol/l) concentrations is essentially unaffected by adenoviral-mediated overexpression of LDH-A. Similarly, glucose-induced $[Ca^{2+}]_c$ increases are unaffected under these conditions. These data therefore suggest that the previously observed inhibitory effects on insulin secretion stimulated by 30 mmol/l glucose from stably transformed MIN6 β-cells (9) may be in part the result of changes in the expression of other genes. Nevertheless, and in agreement with the finding of diminished U-^{14}C glucose oxidation and enhanced lactate output in LDH-A overexpressing INS-1 cells (34), oxidative glucose metabolism was significantly inhibited under these conditions, as judged by changes in a panel of mitochondrial metabolic parameters (Fig. 3). The simplest explanation of this dichotomy between stimulated secretion and metabolism is that, under these conditions, the generation of coupling factors such as ATP is no longer limiting for activated secretion.

The present studies also reveal that at intermediate glucose concentrations, stimulation of both insulin secretion and $[Ca^{2+}]_c$ increases is strongly inhibited by the overexpression of LDH-A. Thus, elevated LDH-A activity appears to induce a "right-shift," or desensitization, in the response to glucose. Under these conditions, generation of coupling factors by mitochondrial metabolism may be more limiting of insulin secretion compared with conditions of acute glucose stimulation. Importantly, such a desensitization is believed to underlie the characteristic hyperglycemia observed in type 2 diabetic patients. These results also suggest that it may be important in future studies where the expression of islet enzymes is altered to investigate the effects at intermediate, as well as at low and high (maximal), glucose concentrations.

In addition, LDH-A overexpression caused a dramatic alteration in the response of MIN6 cells to prestimulation with submaximal glucose concentrations. Whereas preperifusion at 11 mmol/l glucose had no detectable effect on the subsequent secretory response observed when the glucose concentration was raised to 30 mmol/l (~6-fold in each case), cells preperifused at intermediate glucose concentrations displayed a dramatically weakened response to 30 mmol/l glucose. These data suggest that during the preincubatory period, LDH-A+ cells experience inhibitory changes that later impact during stimulation at high glucose concentrations. At present, the nature of these changes is obscure. Although increased acidification of the cells represents one possibility, it might be noted that this was not observed after stable overexpression of MIN6 cells with LDH (8). Further examination of this question will require detailed metabolic studies with LDH-A–infected islets, as previously performed in uninfected islets (6).

It is noteworthy that the observed differences in metabolism in LDH+ cells were more pronounced >250 s seconds after stimulation with glucose. This coincided with the second phase of mitochondrial metabolism, which followed an initial rapid "burst" phase most clearly seen in the NAD(P)H response. Because the onset of phase II followed the onset of increase of $[Ca^{2+}]_c$ and because calcium is a known activator of mitochondrial dehydrogenases (28,31,32,35), it is therefore possible that phase II was a consequence of $[Ca^{2+}]_c$, and subsequently $[Ca^{2+}]_m$, increases that led to activation of mitochondrial metabolism. Indeed, the abolition of this second phase by Ca^{2+} removal (Fig. 5*B*) is consistent with this self-amplification model.

In addition to ensuring predominantly oxidative metabolism of glucose, suppression of LDH expression in native β-cells may be important in ensuring that lactate does not act as an insulin secretagogue, for example during exercise or starvation. Lactate and pyruvate could potentially generate coupling factors, such as ATP, if they were oxidized in the β-cell. It should be noted that although isolated islets do, in fact, oxidize pyruvate and lactate readily (1), the contribution of β-cells to this metabolism is uncertain, and may be small. Hence, low LDH and monocarboxylate transporter activities in the β-cell would afford "protection" from activated insulin secretion during exercise. Recent evidence (33) from studies in INS-1 cells demonstrates that co-overexpression of both these activities results in stimulation of secretion by lactate and pyruvate. Interestingly, a case has been reported of an individual in whom exercise leads to acute hypoglycemia and hyperinsulinemia (T. Otonkoski, personal communication). An intriguing possibility is that inappropriately high expression of LDH, lactate transport activity, or both may permit lactate-stimulated insulin secretion in this patient.

Future studies are required to determine whether alterations (increases) in LDH-A expression in islets have effects on glucose metabolism similar to those observed in MIN6 cells and if they are associated with common forms of type 2 diabetes. Analysis of linkage between the promoter region of the LDH-A and -B genes (located on chromosome 11) and monogenic familial diabetes (maturity-onset diabetes of the young) (36) may also be instructive.

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