Histochemical Evidence for Pathways Insulin Cells Use to Oxidize Glycolysis-Derived NADH

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The activity of lactate dehydrogenase is known to be low in the pancreatic β cell, and the activity of the mitochondrial glycerol phosphate dehydrogenase (mGPD), the key enzyme of the glycerol phosphate shuttle, is known to be high in this cell. Lactate dehydrogenase was demonstrated histochemically in insulin positive cells of the rat pancreas, and its activity was semi-quantified densitometrically; activity in these cells was estimated to be about 8% of that in the surrounding acinar tissue. mGPD histochemical activity was extremely high in cells exhibiting insulin immunofluoresence, while activity in surrounding pancreas tissue was negligible. When the activity was measured in situ at a physiologic concentration of substrate, this enzyme was inactive in the absence of free calcium. These results are consistent with the idea that glucose, the most potent physiologic insulin secretagogue, stimulates insulin secretion via aerobic glycolysis. If glycolysis-derived NADH, instead of being reoxidized in a mitochondrial hydrogen shuttle, is reoxidized to NAD by pyruvate via the reaction catalyzed by lactate dehydrogenase with the resulting formation of lactate, there would be little or no pyruvate available for mitochondrial metabolism. Consequently adenosine triphosphate formation would be about 5% to 7% of that formed by the complete combustion of glucose to carbon dioxide via mitochondrial metabolism. The low lactate dehydrogenase and high mGPD emphasize the importance of mitochondrial hydrogen shuttles for reoxidation of glycolysis-derived NADH in insulin secretion.

GLUCOSE IS THE MOST potent metabolizable insulin secretagogue, and it stimulates insulin secretion from pancreatic β cells by aerobic glycolysis. A great deal of evidence suggests that β cells possess high levels of enzymes that participate in hydrogen shuttles that oxidize NADH formed in the cytosol during glycolysis. Because the inner mitochondrial membrane is impermeable to NAD(H), hydrogen shuttles have evolved to transport reduced substrates (in)to the mitochondria where they are oxidized. The oxidized substrates return to the cytosol to oxidize NADH. The levels of enzymes for 2 such shuttles, the glycerol phosphate shuttle,[1-5] and the malate aspartate shuttle,[6] are very high in the β cell, and these high enzyme levels, as well as metabolic studies,[5,7-9] suggest that hydrogen shuttles are important for insulin secretion.

It is somewhat surprising, however, that β cells, unlike other tissues, such as muscle, which can metabolize glucose rapidly, probably do not reoxidize NADH formed in the cytosol by a system that is, perhaps, simpler and more prevalent in many types of cells. That is by utilizing the lactate dehydrogenase reaction and the Cori cycle in which pyruvate oxidizes NADH to NAD and is converted to lactate, which is exported from the cell to be oxidized in another tissue, such as liver. Because the total mass of β cells relative to body mass is extremely small in any mammal, this would not place significant redox stress on the organism and would seem an efficient means of handling reducing equivalents in the β cell cytosol. However, this system seems to have not evolved in the β cell. Several histochemical studies suggest that the level of lactate dehydrogenase is low in β cells, however, its activity was not quantified.[10-18] The above facts suggest that the β cell maintains a normal cytosolic redox state via mitochondrial hydrogen shuttles. To obtain more clues about this hypothesis, histochemical studies of lactate dehydrogenase and mitochondrial glycerol phosphate dehydrogenase (mGPD), the key enzyme of the glycerol phosphate shuttle, in the insulin cell were performed.

It is known that calcium can activate the mGPD when the enzyme's activity is estimated in a standard enzyme reaction mixture[19] (and references 1 to 9 therein). However, calcium's effect on the enzyme has never been demonstrated in situ histochemically. The current study shows calcium can activate mGPD in situ in β cells in sections of pancreas and also that the enzyme activity of mGPD in β cells is high as judged from histochemistry assays. The histochemical enzyme activity of lactate dehydrogenase was quantified by densitometry and shown to be 8% of that in pancreatic acinar tissue.

MATERIALS AND METHODS

Histochemistry

Pairs of fresh frozen sections (8 μm thickness) were made from pancreata removed from Sprague Dawley rats weighing 350 g and mounted on gelatin coated slides. The first section of each pair was fixed in 4% paraformaldehyde for 15 minutes, rinsed in phosphate-buffered saline (137 mmol/L NaCl, 3 mmol/L KCl, 10 mmol/L Na_2HPO_4, and 2 mmol/L KH_2PO_4) (PBS) for 5 minutes and blocked with PBS containing 5% goat serum, 0.3% Triton-X 100, and 0.05% sodium azide for 15 minutes and then rinsed in PBS for 2 minutes. Mouse anti-insulin monoclonal antibody (1:800 dilution) (Sigma [St Louis, MO] catalog number 1-2018) was applied to the tissue for 3 hours at room temperature. The tissue was rinsed in PBS for 10 minutes and a fluorescein-isothiocyanate conjugated goat antimouse monoclonal antibody (Sigma F-2012) (1:100 dilution) was applied for 60 minutes at room temperature in the dark. The slide was rinsed in PBS for 2 minutes and photographed with a Nikon U FX-II camera system (Tokyo, Japan).

The second slide of the pair was used to histochemically localize both lactate dehydrogenase and mGPD. First, lactate dehydrogenase activity was estimated by incubating the slide in 20 mmol/L lactate, 1 mmol/L NAD, 2.5 mmol/L nitroblue tetrazolium, 0.25 mmol/L phena-

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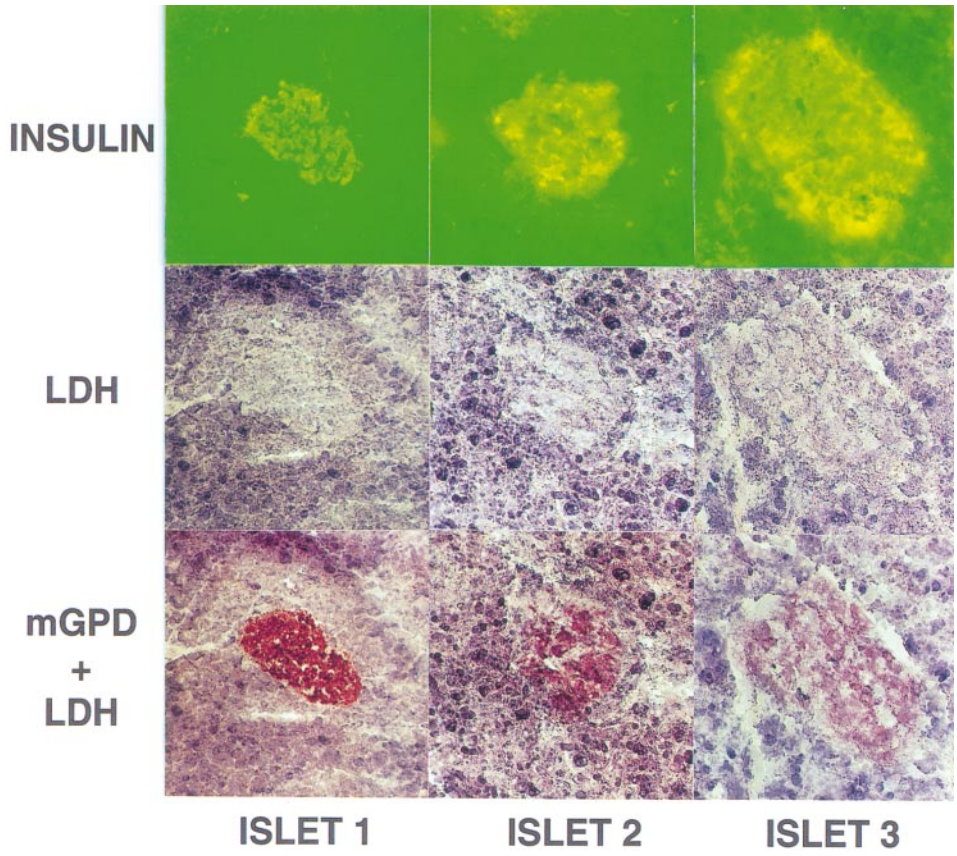
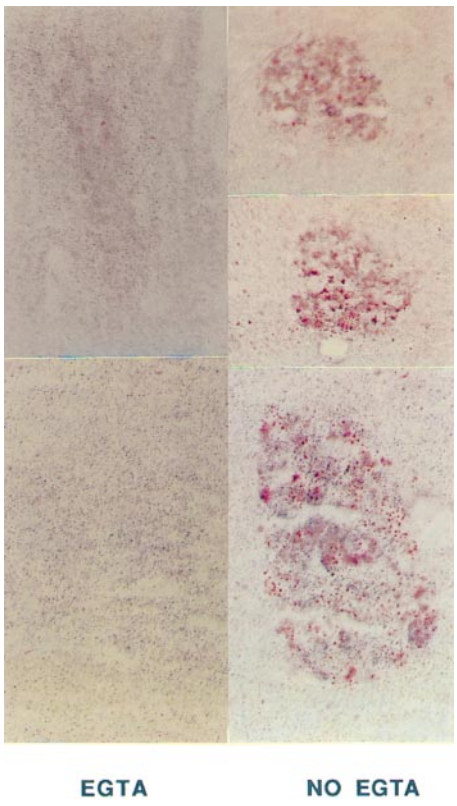


Fig 1. Colocalization of insulin, low lactate dehydrogenase activity, and high mGPD activity in pancreatic β cells. Lactate dehydrogenase activity was developed in a frozen section of rat pancreas, photographs were taken, and then mGPD activity was developed and the sections rephotographed as described in Materials and Methods. In a neighboring serial section, insulin was identified by immunofluorescence. Original magnification × 94.



zine methosulfate, 5 mmol/L KCN, 1% agarose, and 80 mmol/L Tris-chloride buffer, pH 7.5, for 40 minutes at 37°C.[20,21] The slide was then rinsed in PBS and photographed. After the lactate dehydrogenase reaction was photographed, the same section of tissue was used to localize mGPD enzyme activity. mGPD activity was estimated by incubating the slide in a mixture containing 50 mmol/L L-glycerol-3-phosphate as its D-L isomers, 4 mmol/L iodonitrotetrazolium violet, 10 mmol/L KCN, and 50 mmol/L Bicine buffer, pH 8.0 at 37°C for 15 minutes and photographed.[1-3,21] The density of the lactate dehydrogenase reaction in photographs was quantified with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA). Each enzyme activity was measured in more than 20 sections from 6 or more pancreata.

In a separate set of experiments, activation of β cell mGPD by calcium was demonstrated in situ by incubating fresh frozen sections of rat pancreas in a reaction mixture identical to the one above except that the concentration of L-glycerol-3-phosphate was 50 μmol/L.[21] When EGTA (2 mmol/L) was present in the reaction mixture, it was added 10 minutes before the substrates. After the difference in staining between the sections incubated with and without EGTA was readily apparent (about 45 to 60 minutes), the reaction mixture was removed and the slides were fixed in 5% glutaraldehyde in 100 mmol/L Tris-chloride buffer, pH 8, for 5 minutes, rinsed in water, and photographed.

Fig 2. Calcium activation of mGPD in the pancreatic β cell. Frozen sections of rat pancreas were incubated with EGTA to chelate calcium (left panels) or without EGTA (right panels) as described in Materials and Methods. The distribution of enzyme activity in the central portion of the islet conforms to the known distribution of β cells and insulin staining in the islet as seen in Fig 1. Original magnification × $\simeq$ 94.

RESULTS AND DISCUSSION

Figure 1 shows photographs of 3 separate pancreata stained for lactate dehydrogenase and mGPD enzyme activity, as well as insulin immunoreactivity. The activity of lactate dehydrogenase is clearly much lower in the insulin-staining cells of the islet than in the surrounding acinar tissue of the pancreas, while the mGPD activity is much higher in islets than in the acinar tissue consistent with reported high assayable mGPD activity in isolated pancreatic islets and low activity in whole pancreas.[1-3] Quantification of the density of staining due to lactate dehydrogenase in the islet indicated that it was 8% ± 2% (mean ± SE, n = 3) that of the surrounding acinar tissue. The very dark staining of mGPD activity in the islet compared with the acinar tissue is in good agreement with earlier estimates that islet mGPD exceeds that of whole pancreas and many other tissues of the body by 40- to 70-fold.[1]

mGPD is activated by calcium via its directly binding to the enzyme[19] (and references therein). Figure 2 demonstrates that using EDTA to chelate calcium normally present in the pancreatic tissue and/or in laboratory reagents almost completely prevents calcium's activation of mGPD in the islet. During stimulation of the islet with an insulin secretagogue, such as glucose, the concentration of calcium in the β cell increases to that which permits one half maximal activation of mGPD (about 0.1 μmol/L).[5] This should permit mGPD to more rapidly reoxidize the increased NADH resulting from the increased rate of glycolysis.[6,19]

The high mGPD and its activation by calcium plus the low lactate dehydrogenase level in the β cell provide clues about the metabolism of glucose in the β cell. It was recently reported that the β cell plasma membrane, unlike that of many other cells, contains very little lactate transporter activity, suggesting that lactate cannot be rapidly exported from the β cell.[22,23] However, the low level of lactate dehydrogenase in the β cell must be sufficient to maintain the lactate/pyruvate ratio which, along with the glycerol phosphate/dihydroxyacetone phosphate ratio buffers the cytosolic NAD/NADH ratio. For every molecule of pyruvate formed during glycolysis that is converted to lactate and is exported to the circulation, the energy derived from that particular pyruvate comes only from anaerobic glycosis. Anaerobic glycolysis produces only 2 molecules of adenosine triphosphate (ATP) per molecule of glucose metabolized to pyruvate. In contrast, during aerobic glycolysis in which pyruvate enters the mitochondrion and is decarboxylated to acetyl-CoA, which is completely combusted to carbon dioxide (CO_2), 36 to 38 molecules of ATP are produced per molecule of glucose. In addition, pyruvate lost to mitochondrial metabolism could not be carboxylated to oxaloacetate by pyruvate carboxylase in the mitochondria. The resulting 4 carbon dicarboxylic acids participate in a number of anaplerotic reactions,[24,25] including the pyruvate malate shuttle, which exports NADPH equivalents and CO_2 from the mitochondrial matrix to the cytosol.[24]

Some of the ATP formed in the complete combustion of glucose is derived from the oxidation by hydrogen shuttles of glycolysis-derived NADH. Every triose phosphate metabolized in glycolysis results in 1 molecule of NAD reduced to NADH. If this NADH is oxidized in the glycerol phosphate shuttle, 2 molecules of ATP are formed per triose phosphate, and if an NADH equivalent is oxidized via the malate aspartate shuttle, 3 molecules of ATP are formed per triose phosphate. Thus, hydrogen shuttles provide 4 or 6 additional molecules of ATP per molecule of glucose metabolized via glycolysis. Besides possessing high levels of enzymes of the glycerol phosphate shuttle,[1,6] the β cell also possesses high levels of enzymes of the malate aspartate shuttle.[6]

Although the current study may be the first to quantify the histochemical activity of lactate dehydrogenase in the β cell, there is at least 30 years of histochemical evidence to suggest that lactate dehydrogenase in the pancreatic islet is low.[10-18] The low levels of lactate dehydrogenase and lactate transporter[22,23] and high amounts of hydrogen shuttle enzymes[1-6,19,22] in the β cell support the idea that glucose is oxidized aerobically in the β cell to maximize energy production. Because glucose metabolism causes an increase in cytosolic calcium levels in the β cell, calcium's activation of mGPD will permit the glycerol phosphate shuttle to more rapidly reoxidize glycolysis-derived NADH.

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