Citrate Oscillates in Liver and Pancreatic Beta Cell Mitochondria and in INS-1 Insulinoma Cells*

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Michael J. MacDonald‡, Leonard A. Fahien, Julian D. Buss, Noaman M. Hasan, Michael J. Fallon, and Mindy A. Kendrick

From the Childrens Diabetes Center, University of Wisconsin Medical School, Madison, Wisconsin 53706

Oscillations in citric acid cycle intermediates have never been previously reported in any type of cell. Here we show that adding pyruvate to isolated mitochondria from liver, pancreatic islets, and INS-1 insulinoma cells or adding glucose to intact INS-1 cells causes sustained oscillations in citrate levels. Other citric acid cycle intermediates measured either did not oscillate or possibly oscillated with a low amplitude. In INS-1 mitochondria citrate oscillations are in phase with NAD(P) oscillations, and in intact INS-1 cells citrate oscillations parallel oscillations in ATP, suggesting that these processes are co-regulated. Oscillations have been extensively studied in the pancreatic beta cell where oscillations in glycolysis, NAD(P)/NAD(P)H and ATP/ADP ratios, plasma membrane electrical activity, calcium levels, and insulin secretion have been well documented. Because the mitochondrion is the major site of ATP synthesis and NADH oxidation and the only site of citrate synthesis, mitochondria need to be synchronized for these factors to oscillate. In suspensions of mitochondria from various organs, most of the citrate is exported from the mitochondria. In addition, citrate inhibits its own synthesis. We propose that this enables citrate itself to act as one of the cellular messengers that synchronizes mitochondria. Furthermore, because citrate is a potent inhibitor of the glycolytic enzyme phosphofructokinase, the pacemaker of glycolytic oscillations, citrate may act as a metabolic link between mitochondria and glycolysis. Citrate oscillations may coordinate oscillations in mitochondrial energy production and anaplerosis with glycolytic oscillations, which in the beta cell are known to parallel oscillations in insulin secretion.

The importance of oscillations to biological organisms can be judged from the fact that almost all cells exhibit some kind of oscillations. Oscillations have been extensively studied in the pancreatic beta cell where oscillations in glycolysis, NAD(P)/NAD(P)H and ATP/ADP ratios, plasma membrane electrical activity, calcium levels, and insulin secretion have been well documented (1–5). However, oscillations in citric acid cycle intermediates have never been studied in any system. The beta cell is a unique fuel sensing organ in which mitochondria transduce a metabolic stimulus into multiple pharmacologic stimuli that activate the movement of insulin granules to the plasma membrane and granule extrusion into the circulation (6–9). Glucose, the most potent insulin secretagogue, is metabolized via aerobic glycolysis. Anaplerosis, the synthesis of cycle intermediates, and cataplerosis, the export of cycle intermediates from mitochondria (10), are most likely very important for insulin secretion. In the beta cell about one-half of pyruvate, the terminal product of aerobic glycolysis in the cytosol, enters mitochondrial metabolism via carboxylation through the reaction catalyzed by pyruvate carboxylase, and the other half enters mitochondrial metabolism via decarboxylation catalyzed by pyruvate dehydrogenase (11–15).

Because the synthesis of malate and other four-carbon citric acid cycle intermediates from pyruvate begins with the pyruvate carboxylase reaction is against the direction of citrate synthesis and the citric acid cycle (Fig. 1), this raises an interesting question. How does the synthesis of these four-carbon intermediates proceed simultaneously with citrate synthesis and carbon flux in the cycle unless the synthesis of citrate oscillates out of phase with that of the other cycle intermediates? To address this question we looked for oscillations in several cycle intermediates that are known to be synthesized in large amounts and exported from mitochondria (11, 16–20) by measuring at frequent intervals their levels in preparations of isolated mitochondria from various organs given pyruvate and also in beta cells given glucose. Of all the citric acid cycle intermediates measured, citrate levels exhibited the largest oscillations. Citrate oscillations mirrored NAD(P) oscillations in INS-1 mitochondria and paralleled ATP oscillations in intact INS-1 cells, suggesting that oscillations of these factors are regulated by similar mechanisms.

From the large body of knowledge on the citric acid cycle, it is possible to speculate on the regulation and physiologic significance of citrate oscillations. Because most of the citrate synthesized in mitochondria from pancreatic islets (11, 19) and other tissues (16–18) is exported from the mitochondria and citrate inhibits its own synthesis (21, 22), this may enable citrate oscillations to synchronize mitochondrial metabolism. We further suggest that because citrate is a potent inhibitor of phosphofructokinase (23–26), the enzyme that is believed to be the pacemaker of glycolytic oscillations, citrate can link oscillations in mitochondrial energy production and anaplerosis with glycolytic oscillations for efficient integration of cellular metabolism. This may be especially important in the beta cell where glycolytic oscillations are known to coincide with oscillations in insulin secretion (5). Finally, the data support the idea that the regulation of glucose metabolism in many cells is distributed over many enzyme reactions (27).

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‡ To whom correspondence should be addressed: Rm. 3459, Medical Science Center, 1300 University Ave., Madison, WI 53706. E-mail: mjmacdon@wisc.edu
Citrate Oscillations in Mitochondria and Beta Cells

EXPERIMENTAL PROCEDURES

Mitochondria were isolated by the method of Johnson and Lardy (28) from 30 g of liver of 24 h starved Sprague-Dawley rats; washed three times in 220 mM mannitol, 70 mM sucrose, and 5 mM potassium Hepes buffer, pH 7.5; and suspended in about 30 ml of 215 mM mannitol, 65 mM sucrose, 5 mM potassium Hepes, 5 mM potassium bicarbonate, 5 mM potassium phosphate, 2 mM K$_2$ADP, and 3 mM MgCl$_2$, pH 7.3 (mitochondria incubation medium (MIM))$^1$ (11, 19). Mitochondria were gently stirred in a 100-ml beaker exposed to air at 37 °C. After 35 or 60 min, pyruvate (5 mM) was added, and samples of 0.1 ml of medium were removed at either 15- or 20-s intervals for up to 30 min and rapidly mixed with 0.5 ml of 6% perchloric acid. The perchloric acid extracts were centrifuged, and 0.5 ml of the supernatant fraction was removed and neutralized with about 30% KOH and centrifuged. Perchloric acid pellets were saved for estimates of protein.

Mitochondria were isolated from fed Sprague-Dawley rat pancreatic islets as previously described (11, 19). Mitochondria from about 2000 islets were suspended in 700 μl of MIM and incubated exposed to air at 37 °C. 5 mM pyruvate was added, and samples of 20 μl were taken every 20 s and placed in 3 μl of 6% perchloric acid. The mixture was centrifuged, the pellet was saved for protein measurements, and the supernatant fraction was neutralized with about 3 μl of 30% KOH.

To study metabolite levels, intact INS-1 cells (the 832/13 line developed by Newgard and co-workers (15) or the original line developed by Wollheim and co-workers (29)) were maintained in culture medium as described above but followed by a 90-min culture period in tissue culture medium containing 3 mM glucose and then harvested by trypsinization. The cells (about 0.6 ml of packed cells) were then incubated with gentle shaking at 37 °C in suspension in 4.0 ml of Krebs-Ringer bicarbonate solution, pH 7.3, modified to contain 20 mM sodium Hepes buffer and 20 mM NaHCO$_3$, and containing 0.5% bovine serum albumin. After 15 min 16.7 mM glucose was added, and 50-μl samples were obtained every 15 s. The samples were rapidly mixed with 100 μl of ice cold 6% perchloric acid and kept on ice. After 30–60 min the samples were centrifuged, and 125 μl of the supernatant fraction was removed and neutralized with 14–15 μl of 30% KOH.

Neutralized perchloric acid extracts were used to measure malate, citrate, isocitrate, α-ketoglutarate, and NAD(P) H by alkali-enhanced fluorescence (11, 19, 30) or with native fluorescence (30) in enzymic assays coupled to NAD(P)(H) or with both assays as previously described in detail (11, 19). ATP was measured with luciferase by luminescence (31) with p$^3$P-di (adenosine-5') pentaphosphate in the reaction mixture to inhibit any nucleoside diphosphokinase present. Protein was measured by the Lowry method (32).

To be as certain as possible that citrate oscillations could not be explained by enzyme cycling of metabolites and co-enzymes present in the extract and/or added with reagents, several additional steps in the analysis of citrate were taken. Commercially obtained pure citrate lyase from Aerobacter aerogenes (Roche Applied Science) was further purified by gel filtration on Sephadex G-200, the purity was checked by PAGE, and the purer enzyme was used for citrate assays. In addition, some neutralized perchloric acid extracts from liver mitochondria and intact INS-1 cells were heated at 90 °C for 15 min to inactivate any trace amounts of enzymes possibly not removed by the acid precipitation and/or that might have been reactivated by neutralization of the acid extract. These additional steps in the assay procedure did not change the results.

RESULTS

Preliminary studies suggested that citrate might oscillate in suspensions of mitochondria. Proof of this novel observation required rigorous testing. Therefore, we had different individ-

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$^1$ The abbreviations used are: MIM, mitochondria incubation medium; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
FIG. 2. Oscillations of citrate in the presence of pyruvate in liver mitochondria. Each panel shows a separate experiment. Each experiment was performed by a different individual separated by 2- or 3-month intervals. Fluctuations interpreted to be individual oscillations are separated by vertical dashed lines. A and B show experiments in which mitochondria were incubated in MIM exposed to air for 1 h at 37°C with gentle stirring before 5 mM pyruvate was added at time 0, and sampling was done at 20-s intervals. Citrate was measured by an enzymatic method coupled to alkali-enhanced fluorescence of NAD three times, each time by a separate individual, and the three values were averaged. C and D show
uals perform numerous replicate experiments with both mitochondria and with intact cells every few months for over a year to diminish the possibility that the results were specific to an individual or to seasonal factors. In addition citrate levels were measured by three different individuals, and the values were averaged. We first studied liver mitochondria because they can be readily obtained and, similar to islet mitochondria, possess a high amount of pyruvate carboxylase (11). Although oscillations in many intermediates were either not observed or were small, citrate oscillations were easily demonstrated in liver mitochondria. When pyruvate was added to liver mitochondria, citrate oscillated with the highest amplitude and with a periodicity of $1.5 \pm 2$ min. After about 10 min citrate increased and continued to oscillate (Fig. 2, A–D). Oscillations and an increase in citrate did not occur in the absence of an added substrate or in the presence of uncouplers of oxidative phosphorylation (Fig. 2E). Fluctuations in citrate appeared as true oscillations rather than as random levels because sequential data points were connected in a pattern of ascendance followed by a peak and then descendance and a trough. Malate increased steadily after pyruvate was added, but the amplitude of its fluctuations was small relative to its concentration (Fig. 3), suggesting that it did not oscillate. The levels of α-ketoglutarate and isocitrate were low, and although they appeared to exhibit an oscillatory pattern, fluctuations in their levels were also low making it difficult to discern with certainty that these intermediates oscillated. Most of their data points appeared to be connected in a sequence of peaks suggestive of low amplitude oscillations. However, some regions of data points appeared to be random fluctuations (Fig. 4).

When the microgram amounts of mitochondria obtained from pancreatic islets (Fig. 5) and the INS-1 insulinoma cell line (Fig. 6) were supplied with pyruvate, citrate also appeared to oscillate with a periodicity similar to that observed in liver mitochondria. In addition, citrate also oscillated in all preparations of INS-1 cells given glucose (Figs. 7 and 8). In INS-1 cell mitochondria citrate oscillations coincided with oscillations in NAD(P) (Fig. 6), and in intact INS-1 cells citrate oscillated in

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**Fig. 3. Effect of pyruvate on malate levels in liver mitochondria.** Each panel shows a separate experiment. A–C show malate in the same experiments corresponding to those shown in Fig. 2 (A–C, respectively), in which 5 mM pyruvate was added at zero time. D shows an experiment in which no substrate was added. Malate does not increase in the presence of FCCP, an uncoupler of oxidative phosphorylation (not shown).
parallel with ATP, but offset by a few seconds (Fig. 7). When no substrate was provided to INS-1 cells, fluctuations in citrate were of lower amplitude and less regular than in the presence of glucose (Fig. 7).

**DISCUSSION**

When pyruvate is added to mitochondria from liver, pancreatic islet, or INS-1 cells, sustained large amplitude oscillations in citrate occur. Levels of isocitrate and α-ketoglutarate are low, and if they oscillate, their fluctuations are low in amplitude. Although malate increases more than citrate, it does not appear to oscillate. Citrate also clearly oscillates in INS-1 cells given glucose. Although the main purpose of the current work was to conclusively determine whether citrate oscillates in mitochondria and cells and to obtain data that might explain how the oscillations are regulated, the extensive information available on mitochondrial metabolism makes it possible to accurately speculate about the mechanism of regulation of citrate oscillations and their physiological significance.

An important question is how mitochondria communicate to become synchronized to form the oscillatory citrate pattern. The answer may involve citrate itself and other cycle intermediates. Communication among individual mitochondria is possible because in suspensions of isolated mitochondria, such as from organs as diverse as liver (16, 17), pancreatic islets (11), and heart (18), almost all of the citrate and malate and several other metabolites formed from the metabolism of substrates can be found in the extramitochondrial space. After citrate reaches a high level, its rapid decrease must occur by the uptake of extramitochondrial citrate back into the mitochondria and metabolism in the citric acid cycle as well as by a decrease in its synthesis.

A plausible explanation for the regulation of citrate oscillations can be formulated on the basis of our current data plus what is known from decades of research on the citric acid cycle. Within an individual oscillation, as the citrate level increases and then plateaus (as in Figs. 2, 5, and 6), its rate of synthesis should begin to decrease because citrate synthase is inhibited by its product citrate and also by succinyl-CoA, the product of the α-ketoglutarate dehydrogenase reaction (Fig. 1) (21, 22). In addition, the citrate level should decrease via its metabolism. A high NAD level should favor increased flux through the reactions catalyzed by NAD-isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Fig. 1), thus increasing the rate of citrate metabolism. The fact that the citrate profile exactly mirrors the NAD(P) profile in INS-1 cell mitochondria (Fig. 6) is consistent with this idea. Concomitantly with the metabolism of citrate, the NAD level will decrease from its reduction to NADH, and the NADH level will increase. The resulting lower NAD/NADH ratio will decrease flux through the dehydrogenase reactions. As the citrate and the NAD/NADH ratio reach
their nadirs, the low citrate level will permit its rate of synthesis to increase, and the low NAD/NADH ratio will slow the rate of citrate metabolism. This will contribute to the ascending part of the citrate profile.

The mitochondrial NAD/NADH and ATP/ADP ratios are tightly coupled, and these ratios are known to control flux at various steps of the citric acid cycle (18, 21, 22, 33, 34), including the reaction that synthesizes citrate (Fig. 1). The oscillations in ATP levels in intact INS-1 cells that are parallel with oscillations of citrate (Fig. 7) are also consistent with the idea that ATP/ADP and NAD/NADH oscillations control citrate oscillations or that these factors are controlled by similar mechanisms.

In addition to the citric acid cycle per se regulating oscillations in citrate, pyruvate carboxylase may facilitate citrate oscillations by exerting control strength over the availability of oxaloacetate for synthesis of citrate (Fig. 1). This is possible, if not likely, because the level of oxaloacetate is lower than that of acetyl-CoA and all other cycle intermediates (35, 36) and even lower than the binding sites for oxaloacetate on citrate synthase and other mitochondrial enzymes that react with oxaloacetate, such as malate dehydrogenase and aspartate aminotransferase (37). Thus, the supply of oxaloacetate can be rate-limiting for citrate synthesis.

The reciprocal flow of oxaloacetate and acetyl-CoA into the citric acid cycle also might contribute to an oscillatory pattern in citrate levels. Many conditions that activate pyruvate carboxylase are inhibitory to pyruvate dehydrogenase and vice versa (Fig. 1). High ratios of NADH/NAD, acetyl-CoA/CoA and ATP/ADP inhibit the pyruvate dehydrogenase reaction (38), but ATP is a substrate for pyruvate carboxylase and acetyl-CoA allosterically activates pyruvate carboxylase (39). In addition, pyruvate dehydrogenase is activated by calcium (40), but calcium inhibits pyruvate carboxylase (39). Conversely, a high NAD/NADH ratio favors flux in the malate dehydrogenase reaction in the direction of oxaloacetate and activates pyruvate dehydrogenase (38, 40). This enhances the supply of oxaloacetate and acetyl-CoA that condense to form citrate.

Citrate oscillations may fulfill a requirement in overall cellular metabolism by coordinating mitochondrial energy formation and cataplerosis with oscillations in glycolysis, the pathway that supplies pyruvate for mitochondrial metabolism. Many conditions that activate pyruvate carboxylase are inhibitory to pyruvate dehydrogenase and vice versa (Fig. 1). High ratios of NADH/NAD, acetyl-CoA/CoA and ATP/ADP inhibit the pyruvate dehydrogenase reaction (38), but ATP is a substrate for pyruvate carboxylase and acetyl-CoA allosterically activates pyruvate carboxylase (39). In addition, pyruvate dehydrogenase is activated by calcium (40), but calcium inhibits pyruvate carboxylase (39). Conversely, a high NAD/NADH ratio favors flux in the malate dehydrogenase reaction in the direction of oxaloacetate and activates pyruvate dehydrogenase (38, 40). This enhances the supply of oxaloacetate and acetyl-CoA that condense to form citrate.

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ATP oscillations are in phase with each other. Although their peaks are offset by a few seconds, they sufficiently overlap to provide simultaneous high levels of both citrate and ATP (Fig. 7). High citrate and ATP by inhibiting glycolysis should slow the supply of pyruvate to mitochondria, and this will be coordinated with mitochondrial oxidative metabolism and anaplerosis. This is very likely necessary for efficient cellular metabolism and further supports the idea that the control of metabolic flux in glucose metabolism is distributed among many enzyme reactions (27).

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