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Isolated mouse liver mitochondria are devoid of glucokinase

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Abstract

Glucokinase is a hexokinase isoform with low affinity for glucose that has previously been identified as a cytosolic enzyme. A recent report claims that glucokinase physically associates with liver mitochondria to form a multi-protein complex that may be physiologically important in apoptotic signaling [N.N. Danial, C.F. Gramm, L. Scorrano, C.Y. Zhang, S. Krauss, A.M. Ranger, S.R. Datta, M.E. Greenberg, L.J. Licklider, B.B. Lowell, S.P. Gygi, S.J. Korsmeyer, Nature 424 (2003) 952–956]. Here, we re-examined the association of glucokinase with isolated mouse liver mitochondria. When glucokinase activity was measured by coupled enzyme assay, robust activity was present in whole liver homogenates and their 9500*g* supernatants (cytosol), but activity in the purified mitochondrial fraction was below detection (<0.2% of homogenate). Furthermore, addition of 45 mM glucose in the presence of ATP did not increase mitochondrial respiration, indicating the absence of ADP formation by glucokinase or any other hexokinase isoform. Immunoblots of liver homogenates and cytosol revealed strong glucokinase bands, but no immunoreactivity was detected in mitochondria. In conclusion, mouse liver mitochondria lack measurable glucokinase. Thus, functional linkage of glucokinase to mitochondrial metabolism and apoptotic signaling is unlikely to be mediated by the physical association of glucokinase with mitochondria.

Keywords: Apoptosis; ATP; Cancer; Glucokinase; Hexokinase; Liver; Metabolism; Mitochondria; Respiration

Isoforms of hexokinase (specifically Types I and II) with high affinity for glucose bind physically to voltage dependent anion channels (VDAC) in the mitochondrial outer membrane, where these enzymes gain preferential access to mitochondrially generated ATP [1–3]. In this way, hexokinases Types I and II drive high glycolytic rates typical of rapidly growing tumor cells even under aerobic conditions [4]. Hepatocytes, however, do not express hexokinases Types I, II, and III, but only glucokinase (hexokinase Type IV) [1,5]. Unlike other hexokinase isoforms, glucokinase has low affinity for glucose with an apparent K_m for glucose of 6–10 mM, as compared to ~50 μM for hexokinases Types I, II, and III.

Previously, glucokinase was found in the non-particulate soluble fraction of liver homogenates, and hepatic glucokinase lacks the N-terminal hydrophobic sequence that targets hexokinase I and II to mitochondria [1,6–8]. A recent report, however, claims that glucokinase associates physically with isolated mouse liver mitochondria to form a physiologically important multi-protein complex [9]. This physical association is proposed to link metabolic and apoptotic pathways in liver cells. Mitochondria are not only involved in metabolic pathways, but they also release factors, such as cytochrome *c*, that activate the final and committed phases of apoptosis [10]. Since glucokinase might serve as a link between energetic metabolism and apoptotic signaling, our aim here was to determine whether mitochondria isolated from mouse liver have associated glucokinase, as measured by direct enzymatic assay, immunoreactivity, and glucose-dependent respiratory stimulation. Our results indicate that no glucokinase is present in isolated liver mitochondria to within the sensitivity of our

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assays. Thus, binding of glucokinase to liver mitochondria as occuring with hexokinase Types I and II cannot account for any possible functional linkage mitochondrial metabolism and signaling of apoptosis.

Materials and methods

Materials. Antibodies to glucokinase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sucrose (ultrapure), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, and other reagent grade chemicals were purchased from Sigma Chemical (St. Louis, MO).

Subcellular fractionation. Adult male C57BL/6 black mice (Jackson Laboratory, Bar Harbor, ME) were fasted for 12 h prior to sacrifice by cervical dislocation. In some experiments, fed mice were used. Livers were removed, minced on ice in HE medium (200 mM D-mannitol, 70 mM sucrose, 2 mM Hepes, 0.5 g/L defatted BSA, and 0.5 mM EDTA, pH 7.2) and homogenized (0.3 g of liver/ml of medium) in a glass homogenizer using three strokes of a loose-fitting Teflon pestle. All subsequent steps were carried out at 0–4 °C. Homogenates were centrifuged at 800*g* for 15 min, and the resulting pellets containing nuclei and undisrupted cells were discarded. Supernatants were centrifuged at 9500*g* for 12 min. The resulting supernatants containing cytosol as well as microsomes were called cytosol. Pellets from the second centrifugation were resuspended in H Medium (200 mM D-mannitol, 70 mM sucrose, 2 mM Hepes, and 0.5 g/L defatted BSA, pH 7.2) and centrifuged and resuspended in H medium twice more at 9500*g* for 12 min. The final pellet was resuspended in H medium to 50 mg of protein/ml as the mitochondrial fraction [11].

Mitochondrial respiration assay. Mitochondrial respiration was determined polarographically with a Clark-type oxygen electrode in a closed 1.6 ml glass chamber containing 150 mM sucrose, 5 mM $MgCl_2$, 5 mM Na_2 succinate, 1 μM rotenone, and 10 mM NaPi buffer, pH 7.4, at 23 °C [12]. ADP, glucose, hexokinase, and FCCP were added as indicated.

Glucokinase assay. Glucokinase activity was determined by coupled enzyme assay with glucose-6-phosphate dehydrogenase in medium containing 12 mM $MgCl_2$, 6.5 mM ATP, 1 mM dithiothreitol, 0.9 mM $NADP^+$, 1 IU/ml glucose-6-phosphate dehydrogenase, 45 mM glucose, and 32 mM Na–Hepes buffer, pH 7.6, 22 °C [1]. NADPH formation secondary to glucose-6-phosphate generation by glucokinase was monitored by absorbance at 340 nm assuming a millimolar extinction coefficient of 6.22. To rule out interference due to mitochondrial NAD[P]H production, 4 μM rotenone, 100 nM antimycin A, and 2 μg/ml oligomycin were also added to the assay medium. After addition of homogenate, mitochondria, or cytosol, the assay was initiated with 45 mM glucose. Absorbance was recorded, and the linear rate of absorbance increase over several minutes represented glucokinase activity. Background activity attributable to hexokinase was negligible as measured by running the assay in the presence of 0.1 mM glucose.

Western immunoblotting. Proteins from whole liver homogenate, cytosol, and mitochondria were extracted, resolved by 4–12% SDS–PAGE, and transferred to polyvinylidene difluoride membranes. Immunoblotting was carried out and developed using the ECL Plus kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Signals were imaged using a STORM Image System (Molecular Dynamics, Eugene, OR).

Results

The distribution of glucokinase activity in whole homogenate, cytosol, and thrice-washed mitochondria from mouse livers was measured spectrophotometrically via the coupling of the glucokinase reaction product, glucose-6-phosphate, to formation of NADPH via added glucose-6-phosphate dehydrogenase. Upon addition of glucose, NADPH increased linearly to a nearly identical extent in both homogenate and cytosol (Fig. 1A), and essentially all homogenate glucokinase activity was recovered in the cytosolic fraction (Fig. 1B). By contrast, no glucokinase activity was detected in purified mitochondria to within the limits of detection of the assay (<0.2% of homogenate activity). Exogenous hexokinase added to mitochondria led to robust NADPH formation, which validated the coupled enzyme assay (Fig 1A). We also assessed glucokinase protein levels by Western immunoblotting. Strong glucokinase bands appeared in immunoblots of liver homogenate and cytosol, but no glucokinase immunoreactivity was detectable in immunoblots of mitochondria (Fig. 1A, inset).

The lack of association of glucokinase with purified liver mitochondria was also evident from polarographic traces of mouse liver mitochondria respiring on succinate as substrate (Fig. 2). After addition of 56 μM ADP, mitochondrial oxygen consumption increased (State 3 respiration) until the added ADP was exhausted by conversion to ATP via oxidative phosphorylation, at which point oxygen consumption resumed its slower rate (State 4 respiration). Subsequent addition of 45 mM glucose did not increase the respiratory rate, which should have occurred because of ADP generation if either hexokinase or glucokinase were present (Fig. 2). Subsequent addition of exogenous hexokinase, however, did stimulate respiration. Finally, we added the uncoupler, FCCP, to stimulate a maximal rate of mitochondrial respiration. In other experiments, where 5-times more ADP was added, glucose was still unable to stimulate respiration in the absence of exogenously added hexoki-

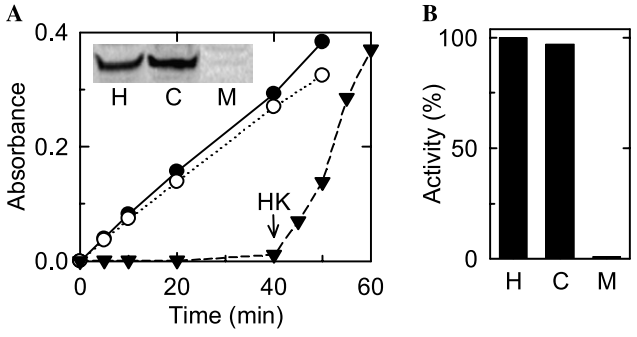

Fig. 1. Glucokinase enzyme activity and immunoreactivity in mouse liver homogenate, cytosol, and mitochondria. (A) Enzyme activity was measured by NADPH formation in a coupled enzyme assay from whole homogenate (●), cytosolic fraction (○), and mitochondria (▼) of samples extracted from the equivalent of 2.5 mg wet weight of fed mouse liver. where indicated, 1 IU of rat hepatoma hexokinase (HK) was added to the M fraction. The inset shows a Western immunoblot (20 μg protein/lane) of homogenate (H), cytosol (C), and mitochondria (M). (B) The relative glucokinase activity in H, C, and M is plotted. Representative of three or more determinations.

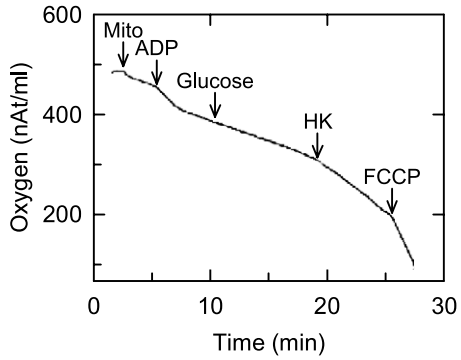

Fig. 2. Absence of glucose stimulation of respiration by mouse liver mitochondria. Shown is a representative polarographic trace of mitochondria (1 mg/ml) respiring on succinate. Where indicated, 56 μM ADP, 45 mM glucose, 1 IU HK, and 6 μM FCCP were added. Representative of three or more determinations.

nase (data not shown). Since glucokinase expression is greater in fed versus fasted mice [13], we compared mitochondria from the livers of fed and fasted mice, but the results were indistinguishable (data not shown).

Discussion

Isoforms of hexokinase (specifically Types I and II) that have high affinity for glucose bind physically to the mitochondrial outer membrane, where they gain preferential access to mitochondrially generated ATP [2,14]. In this way, hexokinases Types I and II drive high glycolytic rates typical of tumor cells even under aerobic conditions [4]. Mitochondria also signal apoptosis and release factors, such as cytochrome *c*, that activate execution of the final and committed phases of apoptosis [10]. Hepatocytes have no hexokinases Types I, II, or III, but only glucokinase [4,7]. Consequently, if glucokinase were to localize to liver mitochondria, it might link metabolic and apoptotic pathways in liver cells, as recently suggested [9].

However, we were unable to measure glucokinase activity or immunoreactivity in isolated mouse liver mitochondria. We employed three separate assays. The first and probably most sensitive was a coupled enzyme assay using glucose-6-phosphate dehydrogenase to couple formation of glucose-6-phosphate by glucokinase (or other hexokinase isoform) to the reduction of $NADP^+$ to NADPH. NADPH formation measured spectrophotometrically revealed that mitochondrial glucokinase was below a detection limit of 0.2% of homogenate glucokinase (Fig. 1). Similarly, we could not detect glucokinase in mitochondrial fractions by immunoblotting (Fig. 1, inset) or by glucose stimulation of coupled mitochondrial respiration (Fig. 2). These findings confirm and extend older reports that hexokinase but not hepatic glucokinase associates with mitochondria of liver and other tissues [1–3,5,8].

Our results would seem to be at variance with [9] in which a similar coupled enzyme assay was used to measure glucokinase activity. In such an assay, a linear steady state rate of NADPH formation signifies the enzymatic activity of glucokinase. However in [9], stepwise increases of NADPH immediately after sample additions were interpreted as enzymatic activity. To the contrary, such increments occurring in just a few seconds and not continuing afterwards do not represent enzyme activity but rather reflect the presence of unidentified metabolites or substrates for glucose-6-phosphate dehydrogenase in the sample. Although, immunoblot data were also presented in [9] in support of the presence of glucokinase in hepatic mitochondria, the putative glucokinase band shown was faint and adjacent to an overloaded lane, which raises the possibility of cross-contamination between electrophoretic lanes or other technical problems. In any event, we were unable to detect immunoreactive glucokinase in thrice-washed mouse liver mitochondria, although abundant glucokinase was present in liver homogenates and cytosol (Fig. 1, inset).

In conclusion, we show that glucokinase in isolated mouse liver mitochondria is negligible and below the limits of detection by the criteria of enzyme activity and immunoreactivity. Consequently, binding of glucokinase to liver mitochondria as proposed recently cannot account for possible functional relationships between mitochondrial metabolism and signaling of apoptosis.

Acknowledgments

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