A sensitive isotopic assay for the measurement of lysine α-ketoglutarate reductase in cultured rat hepatocytes

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Overview

Lysine α-ketoglutarate reductase (LKGR, EC 1.5.1.8) is the rate-limiting enzyme for the catabolism of lysine in mammals and catalyses the reaction:

$$Lysine + \alpha\text{-}ketoglutarate + NADPH = Saccharopine + NADP^{+}$$

Lysine is commonly the limiting amino acid for protein accretion in monogastric animals given cereal-based diets.[1] An understanding of the mechanisms that regulate LKGR activity is therefore of nutritional importance because any unnecessary lysine oxidation is clearly wasteful.

LKGR is a mitochondrial matrix enzyme and its specific activity is highest in the liver, although there is also some activity in other tissues, notably the kidney.[2] When rats are given a diet containing a high lysine content, LKGR activity is significantly increased compared with low lysine-fed controls.[2] In the whole animal it is difficult to determine which factors are directly responsible for changes in enzyme activity, so we have recently developed a cultured hepatocyte model.[3] Using this in vitro system we have shown that the rate of lysine oxidation in cultured rat hepatocytes was modulated by the free lysine concentration of the medium.[3] These observed alterations in rates of lysine oxidation were maximal after 6 hours, suggesting that the observed effect was not purely the result of an increase in substrate concentration, but also a change in LKGR activity.

LKGR activity is typically measured spectrophotometrically using the oxidation of NADPH,[4] or from the accumulation of saccharopine quantified either by amino acid analysis[2,5] or isotopically after separation from the labeled lysine substrate by paper electrophoresis.[6,7] The spectrophotometric assay proved to be unsuitable for our purposes because it was not sensitive enough to assay LKGR activity in small numbers (~2×10^5) of cultured hepatocytes. Although mitochondrial preparations will give a higher specific activity and work well with a spectrophotometric assay, these require the use of large tissue samples. To circumvent these difficulties, we have developed a tracer isotopic assay for use on multiple small samples. This assay can be used to give a rapid and sensitive analysis of the comparative activities of LKGR in hepatocytes cultured in 24-well culture plates; up to 72 samples per day can be assayed by one person.

Principles

Tissue and cell extracts are prepared and incubated in microfuge tubes at 37° C with NADPH and α-ketoglutarate at saturating concentrations (0.69 and 10.8 mmol/L, respectively).[4] L-[U-^{14}C] lysine (6.86×10^5 dpm/nmol lysine) is added at a tracer concentration (1.53 μmol/L) and the reaction allowed to proceed for 6 minutes, during which the reaction rate is linear with time. The reaction is stopped by the addition of sulphosalicyclic acid (SSA), which precipitates cellular protein. After centrifugation, the acid insoluble pellet is assayed for protein and the acid soluble [^{14}C]-lysine and [^{14}C]-saccharopine present in the supernatant are separated by cation-exchange chromatography on short columns of Dowex-50W (sodium form). Radioactivity in saccharopine is determined by liquid scintillation counting. The reaction rate, in DPM/min/μg protein, is converted to moles using the specific activity of the tracer lysine added to the assay.

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Reagents

Distilled and deionized water were used for the preparation of all solutions.

Tissue extraction buffer (220 mmol/L Mannitol, 70 mmol/L sucrose, 2 mmol/L HEPES, 0.05% Nonidet P40, pH 7.4)

The following were weighed and added to ~80 mL water with mixing: 4.01 g Mannitol, 2.40 g sucrose, 0.052 g HEPES. The pH of the solution was adjusted to 7.4 using dilute acid. The buffer volume was then made up to 100 mL with water and 50 μL Nonidet P40 with constant stirring. This buffer was stored at 4° C where it was stable for at least 1 month.

0.1 M HEPES buffer pH 8.3

HEPES (2.60g) was dissolved in 80 mL water with stirring. The pH was adjusted to 8.3 using 0.1M hydrochloric acid and the volume was made up to 100 mL.

0.1 M HEPES buffer containing 0.05% Nonidet P40

This solution was prepared as above except 50 μL Nonidet P40 was added to buffer with stirring before making up to 100 mL.

Phosphate buffered saline pH 7.4

Sodium chloride (9.00 g/L), di-sodium hydrogen orthophosphate (anhydrous, 1.15 g/L) and sodium dihydrogen orthophosphate (0.296 g/L) were dissolved in distilled water. This solution gave a pH of approximately 7.4.

For 50 LKGR isotopic assays

Reagent 1 (43.3 mmol/L α-ketoglutarate in 0.1 M HEPES buffer). α-ketoglutarate (20.6 mg, disodium salt, Sigma, UK) was dissolved in 2500 μL HEPES buffer (pH 8.3).

Reagent (2.7 mmol/L NADPH containing U-^{14}C lysine in HEPES buffer). NADPH (6 mg, tetrasodium salt, Sigma) was dissolved in 2460 μL 0.1 M HEPES buffer pH 8.3. After dissolution 40 μL of U-^{14}C lysine (Amersham, Arlington Heights, IL USA specific activity 11.5 GBq/mmole) was added to the solution and mixed.

Column type

The columns used were 10 mL BioRad Poly-Prep (BioRad Laboratories, Richmond, CA USA) chromatography columns.

Dowex 50W cation-exchange resin

The resin was regenerated in the first instance and altered to the sodium form by mixing a quantity of resin (approximately 1.5 mL per column) with 300 mL of 2 M NaOH (80 g/L) using a glass rod. The mixture was heated at 60° C for 5 minutes. The regenerated resin was then transferred to a Buchner funnel on top of a vacuum flask where the resin was drained under vacuum. The Dowex was then washed with 2 × 300 mL distilled water. After washing, the resin was resuspended in a small volume of water and applied to the columns using a large syringe. Approximately 1.5 mL of resin was applied to each 10 mL column (BioRad Poly-Prep chromatography column). The columns were then washed with 10 mL 0.2 M tri-sodium citrate (19.6 g/L), pH 2.2 until the eluant pH was 2.2. For best results the columns were regenerated immediately prior to use. After the elution of saccharopine, the column-bound lysine was eluted by washing the column with 10 mL 2 M NaOH. The columns can be re-equilibrated with 0.2 N tri-sodium citrate buffer (pH 2.2) for further separations. Using this regeneration procedure the resin was stable for at least 6 months and was stored in 0.1 M NaOH (4 g/L) to prevent microbial contamination.

Procedures

Preparation of LKGR from cultured hepatocytes

Hooded Lister rats (Rowett strain, 200–250 g body weight) were anesthetized using sodium pentobarbitone (150 μL/200g rat body weight) and the hepatic portal vein was catheterized. Hepatocytes were isolated using a two-step collagenase perfusion.[8] Isolated hepatocytes (2 × 10^5) were transferred to each well of a 12-well plastic tissue culture plate (Linbro, well area 4.5 cm^2, Flow Laboratories, VA, USA) containing 250 μL Medium 199 (GIBCO, Grand Island, NY USA)[3] and allowed to attach. At the time of sampling, the medium was aspirated and the attached hepatocytes washed twice with 2 mL phosphate buffered saline (pH 7.4). Five hundred microliters of 0.1 M HEPES containing 0.05% Nonidet P40 (pH 8.3) were then added to each well and the cells were dislodged using a rubber policeman. The cell suspension was then transferred to a microfuge tube and sonicated on ice for 5 seconds. Five to ten μL aliquots of this homogenate were precipitated with 0.5 M Perchloric acid, centrifuged, and the pellet then solubilised in 1 mL 0.3 M NaOH at 37° C for 1 hr. The solubilized protein was assayed using the micro-Bradford method[9] using bovine serum albumin as a standard.

Preparation of LKGR in crude homogenates of rat liver

Hooded Lister rats (Rowett strain, 70–250 g) were killed by cervical dislocation and their livers rapidly excised, blotted dry, and weighed. A weighed subsample of liver (approximately 1 g) was suspended in nine volumes of ice-cold tissue extraction buffer. All the samples were kept on ice unless otherwise stated. The sample was homogenized for 2 × 10 sec using an Ultraturrax homogenizer (Janke & Kunkel, Germany) rotating at 1300 rpm. After homogenization the samples were sonicated for 5 sec. The protein content was determined as detailed above.

LKGR isotopic assay

Using the procedures described above, cultured cell homogenates and crude liver homogenates typically yielded between 2–20 μg protein per μL suspension. Thus, using a known homogenate protein concentration: 1. An appropriate volume of homogenate (equivalent to 20–90 μg protein) was pipetted into labeled microfuge tubes. 2. The total enzyme volume in each microfuge tube was then made up to 100 μL with 0.1 M HEPES/0.05% Nonidet P40. 3. Fifty microlitres of reagent 1 (α-ketoglutarate) was added to the enzyme solution in each microfuge tube and the tubes were incubated at 37° C for 2 min to achieve thermal equilibration. 4. The enzyme reaction was started by the addition of 50 μL reagent 2 (NADPH/^{14}C-lysine) to the enzyme mixture. The tubes were immediately stoppered, mixed, and incubated for 6 minutes at 37° C. 5. The reaction was terminated by the addition of 200 μL 25% SSA. Enzyme blanks containing only the enzyme solution and 50 μL of reagent 2 were run in parallel. At the end of the 6-minute incubation period, SSA was added to the blanks that were then immediately mixed. Fifty microliters of reagent 1 was then added. 6. To precipitate the protein, the tubes were centrifuged at 15,000*g* for 10 min at 4° C in a microfuge. 7. The SSA-soluble supernatant was then aspirated and loaded onto a separate Dowex 50W cation exchange (sodium form) that was previously equilibrated with 0.2 N tri-sodium citrate buffer, pH 2.2 (as described above). 8. The protein precipitates were washed with 1.5 mL 0.2 N citrate buffer pH 2.2 and re-centrifuged as described above. Each citrate wash was then added to its respective sample on the Dowex columns. The SSA-soluble supernatant and tri-sodium citrate washes were allowed to drain through the column; the lysine and saccharopine binding to the column as it drained. 9. Saccharopine was eluted from the column by the addition of 15 mL 0.2 N tri-sodium citrate buffer, pH 3.5. The eluted saccharopine was collected in 20 mL vials. 10. For the scintillation counting, the 15 mL eluant was mixed and a 5 mL aliquot was pipetted into a fresh 20 mL scintillation vial. Ten milliliters Optiphase-X scintillant (LKB, Rockville, MD USA) was added and the vial stoppered, mixed, and counted in a scintillation counter (Packard, Rockville, MD USA; 1900CA). The average counting efficiency was 78%. 11. The SSA-insoluble protein precipitate was solubilised in 1 mL 0.3 M NaOH for 1 hr at 37° C and assayed for protein as described above.

Calculations

Total DPM were calculated from: Total DPM = [CPM(sample) − CPM(blank)]/counting efficiency × 3 (i.e., 5 mL aliquot taken from a total of 15 mL).

LKGR activity was measured as DPM accumulated in saccharopine and expressed per 6-minute period and per μg protein: DPM in saccharopine = Total DPM/6 min incubation period/μg protein

DPM were converted to moles by using the specific activity of the stock [^{14}C] lysine used: moles saccharopine = DPM saccharopine × (1/DPM per mole lysine)

Results and discussion

We describe a reproducible and sensitive assay for measuring hepatic lysine α-ketoglutarate reductase activity. It is sensitive enough to measure LKGR activity in as few as 2 × 10^5 cultured hepatocytes, which is beyond the range of other assay methods. The use of multiple columns (we routinely used 24) enables many samples to be processed simultaneously and allows the effects of many factors to be investigated in multiwell cell culture systems.

In preliminary experiments, the separation of saccaropine (unlabeled) from [^{14}C] lysine on the Dowex columns occurred with 94% recovery of the saccharopine in 15 mL 0.2 N tri-sodium citrate (pH 3.5, *Table 1*). Unlabeled saccharopine was measured in the eluant using an amino acid analyser (Locarte, London, UK).

The pH optima of the assay was 8.3, although LKGR activity had a broad plateau between pH 7.8–8.6 (data not included). An unexplained observation made during these experiments was that the rate of reaction was consistently lower (10–20%) when α-ketoglutarate was added after the NADPH and lysine.

The very high specific activity of this tracer assay allows a very sensitive measure of LKGR at a very low lysine concentration, in this case ~1.53 μmol/L, almost 1400-fold lower than the K_m. At this tracer lysine concentration, the rate of the reaction is directly proportional to the lysine concentration. The other substrates, NADPH and α-ketoglutarate, are at saturating concentrations and therefore do not limit the reaction. When the lysine is added the rate of the reaction is linear for at least 12 minutes *(Figure 1)*.

Table 1 Separation of lysine and saccharopine on Dowex 50W cation exchange media

Eluant pH	Volume eluted (mL)	CPM/mL	Saccharopine recovered (nmol)
2.2	5	18	0
	10	22	0
	15	15	0
3.5	5	7	190
	10	27	224
	15	23	58
			Total = 472
6.1	5	1,996	0
	10	417	0
	15	25	0

One and a half milliliters of Dowex 50W (Na^+) form was equilibrated with 0.2 N tri-sodium citrate buffer, pH 2.2. Samples containing U-^{14}C lysine (16,000 CPM) and 500 nmoles of saccharopine were then loaded onto the column in tri-sodium citrate buffer, pH 2.2. Fifteen milliliters of 0.2 N tri-sodium citrate buffer at three different pH levels (2.2, 3.5, 6.1) were then added to the columns and the eluant was collected in 5 mL samples. The saccharopine eluted was detected using amino acid analysis (Locarte). The recovery of saccharopine was 472/500 × 100 = 94.4 % of the loaded sample (n = 3).

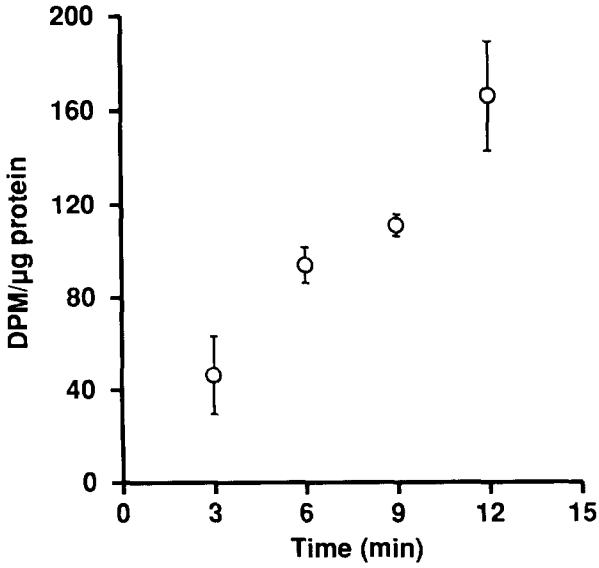

Figure 1 Linearity of lysine α-ketoglutarate reductase activity with time. A 10% (wt/vol) rat liver homogenate was used as described in the text and the reaction was terminated by the addition of 25% sulphosalicyclic acid at 3,6,9, and 12 minutes. The units are DPM/μg protein; $n = 4$ and represent the mean ± 1 SEM.

After this 12-minute period the lysine is depleted and the reaction rate starts to decrease, thus the rate of reaction has to be measured over the initial linear period. We routinely used a 6-min incubation period to ensure that we were within this linear period. The assay is sensitive to the amount of enzyme because the amount of radioactivity in saccharopine increases proportionately as the amount of enzyme is increased *(Figure 2)*.

This assay relies on the use of tracer substrate concentrations, hence unknown amounts of endogenous unlabeled lysine from the tissue/cell extract represents a possible source of interference because the high specific activity of the tracer lysine may be diluted by endogenous free lysine. At very low substrate concentrations, the enzyme rate, v, is proportional to the substrate concentration, [S], so as the addition of unlabeled lysine to the assay decreases the specific radioactivity of the tracer, it also increases the enzyme rate by proportionately the same amount, i.e., doubling the total lysine concentration in the assay halves the specific activity and doubles the rate. Consequently the number of DPM in the product, saccharopine, will be the same irrespective of lysine concentration, although the amount of saccharopine produced will change. Hence, we measure the number of DPM in saccharopine and not the amount of saccharopine produced. To test this theory, unlabeled lysine was added until the lysine concentration exceeded that expected in an assay containing maximal amounts of protein for this assay (i.e., 90 μg hepatic protein, *Figure 3*, see legend for details). *Figure 3* illustrates that there was no significant effect of small amounts of additional lysine on the number of DPM accumulated in saccharopine. Clearly, it is critical that the same amount of DPM as substrate is added to each assay tube.

The number of DPM in saccharopine can be converted to moles using the specific radioactivity of the tracer lysine added. This method calculates the rate if the amount of substrate was derived only from that added as tracer, hence an absolute relationship between enzyme activity and substrate concentration can be established. The fact that the true substrate con-

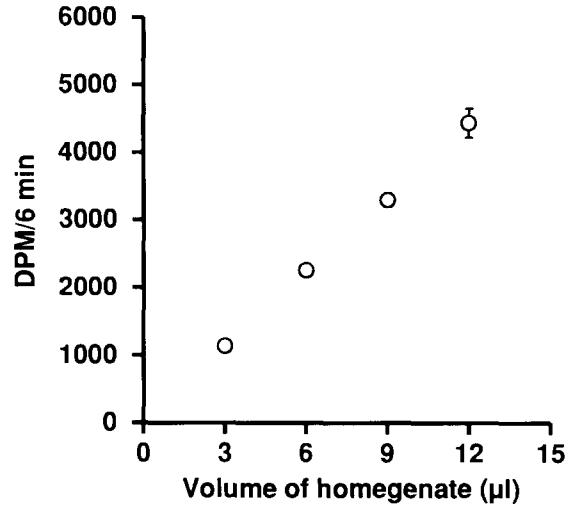

Figure 2 Proportionality of the amount of ^{14}C saccharopine and enzyme volume. A 10% (wt/vol) rat liver homogenate was used as described in the Procedures section. The volume of liver homogenate used was 3–12 μL homogenate and $n = 4$ per point. The results are the mean ± 1 SEM.

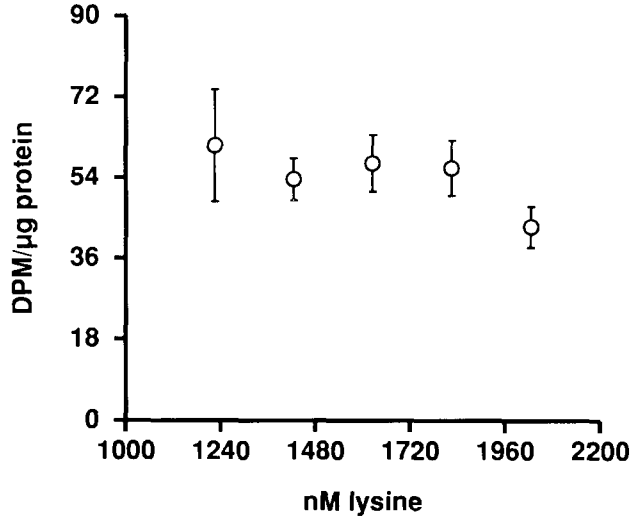

Figure 3 The effect of unlabeled lysine on the LKGR activity (DPM/6 min/μg protein) of isolated rat hepatocytes. A typical LKGR assay (containing 40 μg cell protein) was calculated to have a total of approximately 0.245 nmol lysine/200μL assay (~1225 nmol/L lysine, this included the lysine tracer added and free endogenous lysine). Taking this as a baseline value, the lysine concentration in the assay was increased until it exceeded the protein range over which reliable LKGR activities were observed, i.e., >90 μg protein, which by the same calculations contained a total free lysine concentration of 1,975 nmol/L). A free lysine concentration for liver of 0.003 nmol/μg protein was calculated using published amino acid data[10] and assuming an average protein concentration of 129 mg protein/g liver.[11] The results are the mean ± 1 SEM and $n = 4$.

centration, specific radioactivity, and rate are unknown is irrelevant because changes between them are inexorably interlinked and, as we have shown, do not affect the rate measured in DPM. If the true substrate concentration, specific radioactivity, and rate were determined they would give the same relationship because at these very low substrate concentrations $(k_m \times 10^3)v$ is proportional to the [S].

The further degradation of saccharopine in mammals requires NAD+ and would lead to the loss of labeled saccharopine, amino-adipate, and glutamate being produced further down the degradation pathway. If ^{14}C-saccharopine degradation occurred this would have a significant effect because of the tiny chemical amounts of saccharopine produced. The high concentration of NADPH in the assay will inhibit saccharopine degradation and keep the equilibrium towards saccharopine. The effect of increasing saccharopine concentration on LKGR activity was tested with the rationale that higher saccharopine concentrations should act as a "product trap" diluting the high specific activity saccharopine produced from the lysine tracer. If ^{14}C-saccharopine was further metabolized this would reduce the amount of labeled saccharopine degraded. *Figure 4* shows that even at up to four-fold the saccharopine concentration normally produced in a 6 minute assay, there was no significant effect of saccharopine on the measured rate. Furthermore, where the assay products were analyzed using an amino acid analyzer (Locarte), no production of ^{14}C-labeled glutamate or amino-adipate was observed (data not shown). These results lead us to conclude that the saccharopine produced is not further metabolized.

To compare our measured values of LKGR activity in cultured hepatocytes with in vivo activities, liver homogenates were prepared from rats adapted to a lysine-deficient diet (1%) and a diet excessive in lysine (4%). The calculated V_{max} values (mean, ± SE, n = 4) for LKGR in these rats were 61.9 ± 8.5 and 96.4 ± 9.7 nmol saccharopine produced/min/mg protein,

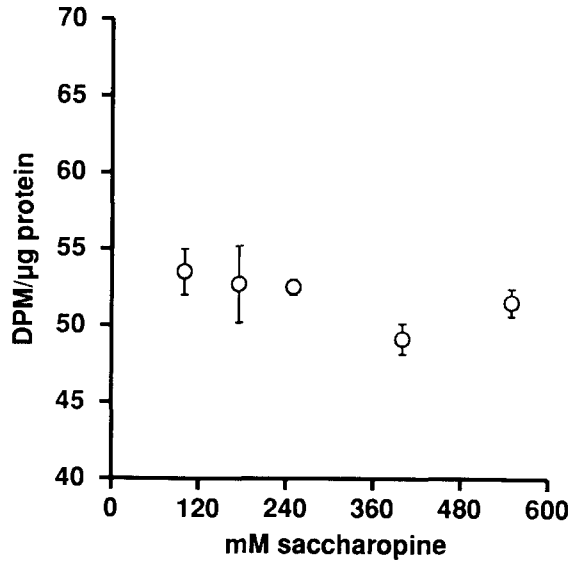

Figure 4 The effect of saccharopine concentration on LKGR activity (DPM/6 min/µg protein) of isolated rat hepatocytes. In a typical 6-minute incubation period, approximately 0.02 nmoles of saccharopine were produced (100nmol/L saccharopine). Taking this value as a baseline, the concentration of saccharopine in the assay was increased approximately four-fold. The results are the mean ± 1 SEM and n = 4.

respectively. These data indicate that this isotopic assay can also be used to measure comparative differences in in vivo LKGR activity. Cultured hepatocytes from rats fed the lysine-excess diet have LKGR activities of 83.5 ± 1.0 nmol saccharopine produced/min/mg protein compared with LKGR activities of 45 ± 1.5 nmol/min/mg protein in hepatocytes from low lysine-fed rats. These data showed that the nutritionally mediated enzyme induction seen in vivo can also be observed in cell culture systems. *Table 2* summarizes the range of literature values for LKGR activity measured using other methodologies and our isotopic assay

Table 2 Summary of LKGR activities reported in the literature

Type of preparation	Type of assay	LKGR	Range of diet type tested	Reference
H	AAA	1.04–2.87	Sucrose/corn starch protein-free diet–similar diet supplemented with 30% lactalbumin	2
H	IPE	0.08–0.64	Potato starch/gluten protein-free diet–similar diet containing 60% casein and supplemented with 3% lysine	7
H	TRACER	61.9–96.4	Lactalbumin based diet deficient in lysine (1%)–similar diet containing excess lysine (4%)	Present study
M	SPEC	84	Laboratory Rat Chow (Oriental Yeast Co., Tokyo)	4
PP	SPEC	28300	Laboratory Rat Chow (Oriental Yeast Co., Tokyo)	4
CH	SPEC	8	Laboratory Rat Chow (Oriental Yeast Co., Tokyo)	12
CH	TRACER	45.0–83.5	Lactalbumin based diet deficient in lysine (1%)–similar diet containing excess lysine (4%)	Present study

LKGR activity is in units of nmol saccharopine produced/min/mg protein. Where necessary an average rat liver protein concentration of 129 mg protein/g tissue[11] was used to convert activity/g tissue into activity/mg protein. Type of preparation = homogenate (H), mitochondria (M), partially purified enzyme (PP), and cultured hepatocytes (CH). Type of assay = amino acid analysis (AAA), isotopic assay followed by separation using paper electrophoresis (IPE), tracer isotopic assay followed by cation exchange chromatography (TRACER), or spectrophotometric (SPEC).

described here. Maximal (V_{max}) LKGR activities with saturating lysine concentrations were calculated for our tracer substrate data by using the Michealis-Menten equation and assuming the K_m for lysine to be 2.2 mmol/L.[4] From the specific activity of the tracer lysine used, the chemical concentration of the lysine used in the assay was then calculated. Thus, using our enzyme assay we obtained LKGR maximal activities that range between 10- and 100-fold higher than the other listed methods *(Table 2)*. However, converting our activities to V_{max} values involves a huge multiplication factor and is sensitive to the assumed K_m value. Also, given the variation in tissue preparation and assay conditions between the methods, these observed differences in measured enzyme activity are perhaps not surprising. It is therefore reasonable that the measurement of enzyme activity in vitro in any assay is unlikely to represent the true activity in vivo and can only be used to assess comparative differences.

In conclusion, the tracer isotopic LKGR method described here has proved to be a consistent and sensitive technique for measuring comparative LKGR activities where low numbers of cells preclude the use of other methods. Furthermore, the sensitivity of this method allows it to be used where only small tissue samples are available (e.g., tissue biopsy). The use of this isotopic essay with hepatocyte cultures will allow us to further study the molecular mechanisms involved in the nutritional and hormonal regulation of lysine α-ketoglutarate reductase in vitro.

Acknowledgments

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