

Late onset of dietary restriction reverses age-related decline of malate–aspartate shuttle enzymes in the liver and kidney of mice

Danswrang Goyary · Ramesh Sharma

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Abstract Dietary restriction (DR) influences several physiological processes, retards the incidences and severity of various age-related diseases and extends lifespan of various animal species. The effect of DR on the activities of malate–aspartate shuttle enzymes, viz. cytosolic and mitochondrial aspartate aminotransferase (c- and m-AsAT) and malate dehydrogenase (c- and m-MDH) was investigated in the liver and kidney of adult (5-months) and old (21-months) male mice. The results show that the activity (U/mg protein) of both c- and m-MDH and AsAT is decreased significantly in the liver and kidney of old mice compared to adult ones. However, DR in old mice reverses significantly the enzyme activities to a level closer to adult animals. Polyacrylamide gel electrophoresis (PAGE) and specific staining of c-AsAT, one of the selected isoenzymes of the shuttle, showed a similar pattern of activity expression as observed by activity measurements in both the tissues studied. Slot blot analysis of c-AsAT confirmed the lower protein content of this isoenzyme in old mice compared to adult ones and a higher level in old-dietary restricted mice. Thus, our results suggest that the late onset of DR in older mice reverses decline in malate–aspartate shuttle enzymes and that it may allow a better metabolic regulation in older animals.

Keywords Dietary restriction · Aging · Malate–aspartate shuttle enzymes · Liver · Kidney · Mice

Introduction

The malate–aspartate shuttle, consisting of mitochondrial (m-) and cytosolic (c-) aspartate aminotransferase (AsAT; EC 2.6.1.1) and malate dehydrogenase (MDH; 1.1.1.37), is a major pathway for the transport of reducing equivalents from cytosol to mitochondria in many animal tissues (Cederbaum et al. 1973; Scholz et al. 1998; Goyary and Sharma 2005). NADH, which is formed during glycolysis in the cytoplasm, is impermeable to inner mitochondrial membrane (Lehninger 1951). Thus, NADH must be regenerated to NAD⁺ for glycolysis to operate. The transfer of reducing equivalents is essential for maintaining a favourable NAD⁺/NADH ratio required for oxidative metabolism of glucose and also for the synthesis of neurotransmitters in the brain (McKenna et al. 2006). The shuttle involves an influx of malate and glutamate and efflux of aspartate and α -ketoglutarate from the mitochondria. Its functional significance is to unfold the degree of control points for glycolysis, gluconeogenesis and Krebs cycle (Borst 1963; Lyngdoh and Sharma 2001). Individual enzymes of various metabolic pathways have been studied in different tissues as a function of age (Kanungo 1980). However, the changes in activity of

D. Goyary · R. Sharma (✉)
Department of Biochemistry, North Eastern Hill
University, Shillong 793 022, India
e-mail: rsharma@nehu.ac.in

enzymes of a particular metabolic pathway are scanty (Sharma 1988). Among mammalian tissues, liver plays an important role in the homeostasis of glucose and a delicate balance exists between absorption of dietary carbohydrates by the intestine and the rate of glucose utilization by tissues, its release by the liver and its reabsorption through kidney tubules. Thus, several important pathways in the liver and other tissues play pivotal roles in the control of glucose homeostasis by maintaining a balance between its uptake/storage by glycogenesis, and release by glycogenolysis/gluconeogenesis (Hagopian et al. 2003).

Dietary restriction (DR), i.e. a reduction in calorie intake without malnutrition, has been known to influence several physiological processes viz., immunological, protein and amino acid metabolism and also neuroendocrinological system (Windruch 1996; Pahlavani 2000). It is an efficacious mode to increase longevity and delay the incidence and severity of various age-associated pathologies including cardiomyopathy, nephropathy and spontaneous and chemically induced tumorigenesis (Roth et al. 2000; Colin et al. 2003; Sharma 2004). DR is known to extend the mean and maximum lifespan in numerous organisms from yeast to rodents and possibly primates (Lin et al. 2000; Nisoli et al. 2005; Willcox et al. 2006). It evokes anti-inflammatory, antineoplastic effects and also protects aging rodents against diabetes, impaired tissue growth and reproductive senescence (Lynn et al. 1998). DR is known to enhance apoptosis in mitotic tissues, resulting in more efficient removal of preneoplastic and neoplastic foci (Hursting et al. 2003). It also enhances longevity by potentiating the immune responses and lowering the oxidative stress/damage (Colin et al. 2003; Sharma 2004). Studies in rats have shown a decrease in protein turnover with advancing age (Lewis et al. 1985; Obled and Arnal 1991). However, it has been observed that DR promotes longevity through metabolic reprogramming with a transcriptional shift towards reduced energy metabolism and increased biosynthesis and turnover of proteins (Weinert and Timiras 2003). DR significantly increases gluconeogenesis that concurs with an increased rate of protein turnover during such intervention (Hagopian et al. 2003). It has also been reported that the damaged protein accumulation plays a major role in aging and the ability of the system to

remove such proteins could be enhanced by DR (Van Remmen et al. 1995). As the role of malate–aspartate shuttle is very crucial in the oxidative metabolism (Minarik et al. 2002), we studied the level of malate–aspartate shuttle enzymes in the liver and kidney of adult, old and old mice subjected to DR.

Materials and methods

Animals and diet

Swiss albino (Balb/C strain) male mice of two different age groups (adult, 5-months and old, 21-months) maintained under normal laboratory conditions were used. They were fed with a standard pellet diet (Amrut Laboratory, Pune) and water *ad libitum* as per experimental schedule. A group of old mice was fed on alternate days for a period of three months (Merry 1999; Goyary and Sharma 2005). Animals were sacrificed at the end of a feeding day. Mice maintained on such regimen are known to consume 30% less food over a period of time and live up to 30% longer, compared to *ad libitum* fed animals (Lee et al. 2000).

Chemicals

NADH, oxaloacetic acid, aspartic acid, α -ketoglutarate, malate dehydrogenase, tris-base, fast blue B salt (O-Dianisidin-tetrazotized), TEMED, ammonium per sulfate, coomassie brilliant blue R-250, bromophenol blue, acrylamide, bisacrylamide and nitrocellulose membrane were purchased from Sigma Chemical Co., USA. All other chemicals used were of analytical grade.

Buffers

Following buffers were used: (A) 0.1 M potassium phosphate buffer, pH 7.5, (B) 10 mM potassium phosphate buffer, pH 7.5/0.25 M sucrose/0.5% triton X-100, (C) 50 mM potassium phosphate buffer, pH 7.5 containing 0.25 M sucrose, (D) Tris-glycine buffer pH 8.3, (E) TBS buffer (20 mM Tris-HCl, pH 7.5/500 mM NaCl), and (F) TTBS (20 mM Tris-HCl, pH 7.5/500 mM NaCl/0.05% Tween 20).

Tissue preparation

Mice were sacrificed by cervical dislocation at a fixed time of the day (13:00 h) and their liver and kidney were taken out, washed in chilled normal saline (0.9% NaCl) and blotted dry. A 10% (w/v) homogenate of these tissues was prepared in ice-cold 0.25 M sucrose solution using a homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at 800g for 10 min at 0°C to sediment nuclei. The resulting supernatants were further centrifuged at 14,000g for 30 min at 0°C to sediment mitochondria. The supernatants thus obtained were used for the assay of c-AsAT and c-MDH. The mitochondrial pellet was washed twice and suspended in a solubilizing medium. For the assay of m-AsAT, the mitochondrial pellet was suspended in buffer B and for the assay of m-MDH, it was suspended in buffer C. Assays were performed within 3 h of mitochondrial suspension.

Assay of shuttle enzymes

The activity of c- and m-AsAT was measured as described (Karmen 1955), with some modifications (Herzfeld and Greengard 1971; Sharma et al. 1992). The c- and m-MDH were assayed spectrophotometrically as described (Kitto 1969) with certain modifications of our own (Goyary and Sharma 2005). The rate of oxidation of NADH was measured at 340 nm using a cuvette of 1.0 cm light path. The activities of both isozymes of AsAT and MDH were expressed as units ($\mu\text{mole NADH oxidized per min}$) per mg protein at 25°C. Protein content of the enzyme preparation was measured according to the dye-binding method of Bradford (1976) using bovine serum albumin as standard. Data obtained from different sets were analyzed using Student's *t*-test and the level of significance ($P < 0.05$) between two sets of data was taken as significant.

Polyacrylamide gel electrophoresis of c-AsAT

Native polyacrylamide gel electrophoresis (PAGE) of c-AsAT, one of the selected isoenzymes of the shuttle, from the liver and kidney of adult (A), old (O) and old-dietary restricted (O-DR) mice was

performed according to the method of Davis (1964) with slight modification. To each gel lane, 30 μl of cytosol adjusted to equal protein (100 μg) from adult, old and old-dietary restricted (O-DR) mice was applied. After the electrophoresis in cold, gel was processed for specific staining of c-AsAT according to the method of Doonan et al. (1981) with certain modifications (Sharma and Patnaik 1982). The staining mixture contained L-aspartic acid (15 mM), α -ketoglutarate (6.8 mM), and tris (100 mM, pH 7.5). Prior to use, O-Dianisidine-tetrazotized (Fast Blue B Salt) was added to a final concentration of 10 mM and staining mixture was stirred vigorously. The mixture was then poured on to slab gel and the enzyme activity band appeared as a violet colour within a short span of time. The staining mixture was then decanted and the gel was washed thoroughly to avoid any further development of background colour. These gels were stored overnight in a solution of distilled water, methanol and acetic acid in the ratio of 5:3:1 (v/v) and subsequently scanned in hp Scan jet 7400C.

Slot blot analysis

The blotting was performed on a Bio-Rad Bio-Dot® SF Microfiltration apparatus following the instructions given in the user's manual. A nitrocellulose (NC) membrane (0.45 micron) was soaked for 30 min in buffer E for activation and proper binding. After placing the NC membrane in the slot blot apparatus, the slots used were rehydrated with ddH₂O for uniform binding. To each slot, 100 μl of cytosol adjusted to equal protein (50 μg) from adult (A), old (O) and O-DR mice liver and kidney was applied in the center and was allowed to filter through the membrane by means of a gentle vacuum. The NC membrane was then placed in a blocking solution (5% non-fat milk in buffer E) for an hour. It was then washed twice in buffer F with gentle agitation. After washing the membrane in buffer F, it was again washed twice in buffer E and transferred to a sheep polyclonal to aspartate aminotransferase -(HRP) conjugate (1:2000) solution (abcam plc 332 Cambridge, CB4 0FW, UK, Cat No. ab20577-1) and kept for overnight. The membrane was later washed twice in buffer F and finally with buffer E to remove the detergent. The substrate (TMB/H₂O₂) diluted (1×)

with buffer E was poured over the membrane and after the development of colour, the reaction was stop by washing the membrane in ddH₂O. It was then scanned in hp Scan jet 7400C.

Results

Body weight

The total body weight (g) during alternate days of feeding for 3-months exhibited a significant decrease (–30%; $P < 0.001$) in O-DR mice as compared to old (O) ones (Fig. 1).

Studies of malate–aspartate shuttle enzymes during DR

Our results indicate that mitochondrial and cytosolic malate dehydrogenase (m- and c-MDH) and aspartate aminotransferase (m- and c-AsAT) activity was significantly decreased (–37% and –41% & –28% and –29%) in the liver of old mice compared to adult mice. However, the activity of shuttle enzymes was significantly reversed (+30% and +59% & +66% and +78%) when old mice were subjected to DR for 3-months (Fig. 2a and b). In kidney, c-MDH activity showed no significant change compared to adult mice, however, O-DR mice showed significant increase (+18%) as compared to old unrestricted control. On the other hand, the activity of m-MDH

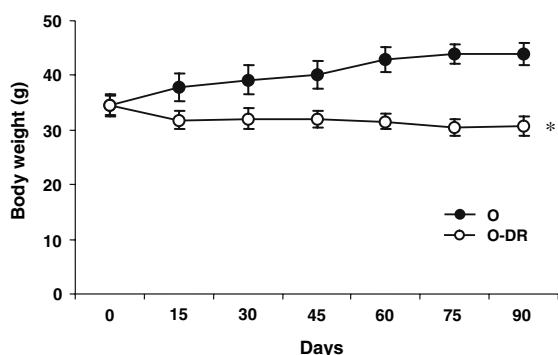


Fig. 1 Body weight of *ad libitum* fed old (O) and old-dietary restricted (O-DR) male mice. Body weight in both groups was measured at 15 days interval for 90 days. Values are expressed as mean from five mice at each point. Bars represent standard deviation. Asterisk (*) represents statistically significant value as compared to old control mice

showed significant decrease (–60%) in old mice as compared to adult ones, however, O-DR mice showed reversal (+94%) of enzyme activity to a level close to adult mice. The activity of m- and c-AsAT decreased (–32% and –34%) significantly in the kidney of old mice as compared to adult ones and got elevated (+94% and +35%) significantly in O-DR mice (Fig. 3a and b).

Polyacrylamide gel electrophoresis and slot blot analyses of c-AsAT

Native PAGE of cytosol containing equal amount of protein from the liver and kidney of adult, old and O-DR mice when stained specifically for c-AsAT, a selected isoenzyme of the shuttle, showed a single band in a pattern similar to the activity level of this isoenzyme as obtained by activity assay measurements (Fig. 4a and b). In both the liver and kidney, it showed a lower intensity in old mice and a higher intensity in O-DR mice. Slot blot analysis also showed a decreased level of c-AsAT protein in old mice liver and kidney compared to adult. O-DR mice exhibited elevated level of this isoenzyme, almost similar to a level of adult mice (Fig. 5a and b).

Discussion

Malate–aspartate shuttle is a pivotal metabolic pathway that allows NADH to gain access to the mitochondrial matrix across impermeable inner mitochondrial membrane. This shuttle provides an important pathway for optimal substrate utilization under conditions of increased metabolic demand (Ralphe et al. 2005). It has been reported that inhibition of the malate–aspartate shuttle results in impaired glucose metabolism and insulin secretion (Bender et al. 2006). Activity of malate–aspartate shuttle enzymes was studied in the liver and kidney of male mice during aging and also an impact of late onset of DR in older mice was assessed on the shuttle enzymes in these tissues. During our experimental schedule, a reduction in body weight was seen in O-DR mice, which confirmed that the mice were indeed subjected to a reduced food intake (about 30%) as observed in previous reports from this laboratory (Dutta and Sharma 2004).

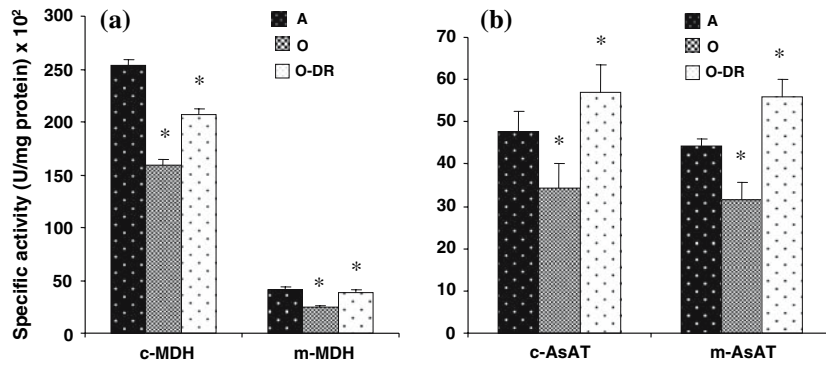


Fig. 2 Activity of cytosolic (c-) and mitochondrial (m-) (a) malate dehydrogenase (MDH) and (b) aspartate aminotransferase (AsAT) in the liver of adult (A), old (O) and old-dietary restricted (O-DR) male mice. Values are expressed as mean

from five mice in each group. Bars represent standard deviation. Asterisks (*) exhibit statistically significant change in old and old-dietary restricted mice as compared to adult and old control mice, respectively

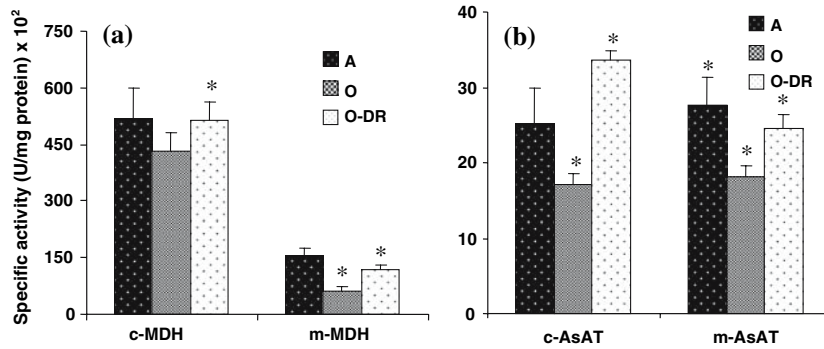


Fig. 3 Activity of cytosolic (c-) and mitochondrial (m-) (a) malate dehydrogenase (MDH) and (b) aspartate aminotransferase (AsAT) in the kidney of adult (A), old (O) and old-dietary restricted (O-DR) male mice. Values are expressed as

mean from five mice in each group. Bars represent standard deviation. Asterisks (*) exhibit statistically significant change in old and old-dietary restricted mice as compared to adult and old control mice, respectively

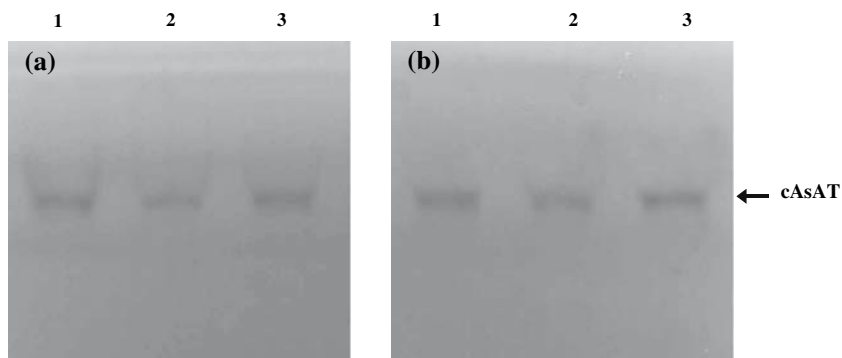


Fig. 4 Polyacrylamide gel electrophoresis (PAGE) of cytosolic aspartate aminotransferase (c-AsAT) from the liver (a) and kidney (b) of adult (lane 1), old (lane 2) and old-dietary restricted (lane 3) male mice. An equal amount (~100 μg

protein) of cytosol containing c-AsAT from the liver and kidney was used. Arrow indicates the position of c-AsAT activity band

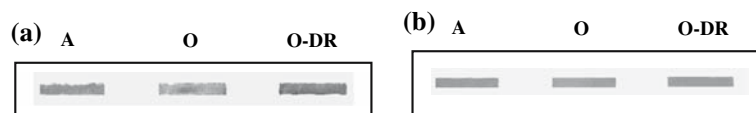


Fig. 5 Slot blot analysis of c-AsAT from the liver (a) and kidney (b) of adult (A), old (O) and old-dietary restricted (O-DR) male mice. An equal amount (50 μ g protein) of cytosol containing c-AsAT was applied on to each slot and processed

for immunoblotting using sheep polyclonal aspartate aminotransferase—(HRP) conjugates. Arrow indicates the content of c-AsAT in respective groups

Our data indicate that c-MDH and AsAT activity declines in the liver with age. Such decline in activities of these isoenzymes is prevented when older animals were subjected to long-term DR. The c-MDH and AsAT are known to be critically involved in the synthesis of glucose from non-carbohydrate precursors (Lardy et al. 1965; Goyary and Sharma 2005). Liver is the major site of gluconeogenesis and thus, decrease in these enzyme activities indicates the reduced gluconeogenesis with age. However, an increased activity of these isoenzymes during DR in older mice may lead to an increased gluconeogenic response when such animals are subjected to DR. This is in agreement with the previous report that c-MDH activity declines with age and that increases significantly in old caloric restricted male C57BL/6J mice (Hagopian et al. 2003). Increased activity of hepatic c-MDH was also reported during caloric restriction from the Fischer 344 rats (Feuer et al. 1989). Our results also indicate that the activity of m-MDH and AsAT decreases with age in mice. These activities significantly increase when old mice were subjected to DR. It is consistent with the earlier findings that calorie restriction induces mitochondrial biogenesis and expression of genes crucial for dynamic processes required for mitochondrial functions (Nisoli et al. 2005).

The kidney too, is known as a second major site of gluconeogenesis. Thus, our findings in kidney on the malate–aspartate shuttle enzymes activity followed the same pattern as does in the liver, except that the c-MDH activity did not change significantly in old mice and this may be due to a differential expression of enzyme in a tissue-specific manner. These findings corroborate with the previous report that the age significantly decreased expression of both glucose 6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA in the kidney (Dhahbi et al. 1999). Our findings on DR of older mice also corroborate with the earlier report that in

late adulthood, acute calorie restriction partially or completely reverses age-related alterations of liver, brain and heart proteins (Spindler 2005). They also reported that the dietary calorie restriction in mice leads to an increase in the mRNA and/or activity of key enzymes (G-6-Pase and PEPCK) of hepatic gluconeogenesis. In an earlier report from this laboratory, it has been shown that an age-dependent decrease in renal glucocorticoid receptor function is reversed by DR in mice. Glucocorticoids are gluconeogenic hormones and their increased function during late onset DR in older animals may provide better adaptability of kidney in such responses (Sharma and Dutta 2006).

The mobility of c-AsAT, one of a selected isoenzymes of malate–aspartate shuttle, from all the three groups (A, O and O-DR) on polyacrylamide gel exhibited a similar migration, and thus indicating that the net charge of the enzyme molecule of the liver and kidney does not change as a function of age and also on DR. Slot blot analyses using sheep polyclonal aspartate aminotransferase -(HRP) conjugate confirmed the decreased level of c-AsAT protein in the liver and kidney of old mice compared to adult and that an elevated level in O-DR ones. These findings confirmed that the decrease in the activity of c-AsAT in the liver of old mice is because of the decline in the expression level of this isoenzyme and that the expression level is elevated in older mice when subjected to DR.

Malate–aspartate shuttle enzymes play crucial role in the transport of reducing equivalents from cytosol to mitosol and are indispensable to citric acid cycle. The decline in the malate–aspartate shuttle enzymes during aging and also increase by late onset of DR corroborates with a recent report that DR initiated later in life provides beneficial effects in reversing some of the biochemical changes during aging (Goto et al. 2007). Thus, our findings suggest that DR even at a later stage in life appears to have better metabolic responses and restore the age-related decline in such functions.

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