

Dietary restriction and triiodothyronine (T₃) regulation of malate-aspartate shuttle enzymes in the liver and kidney of mice

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The activities of malate-aspartate shuttle enzymes viz., cytosolic and mitochondrial aspartate aminotransferase (c- and m-AsAT) and malate dehydrogenase (c- and m-MDH) were measured in liver and kidney of *ad libitum* (AL) and dietary-restricted (DR) mice and also on triiodothyronine (T₃) treatment. The results show that the activity (U/mg protein) of c-AsAT is increased significantly in liver and the activities of c-MDH and m-AsAT are increased significantly in kidney during DR. On T₃ treatment, the activities of both the isoenzymes (c- and m-) of MDH and AsAT are increased significantly in the liver of AL- and DR-fed mice. In the kidney, m-MDH showed no effect by T₃ treatment, however, c-MDH increased significantly in both AL- and DR-fed mice. In contrast, m-AsAT is increased significantly in the kidney in AL-fed mice, but was not affected in DR-fed animals. *In vitro* reconstitution of malate-aspartate shuttle showed a higher activity in the liver and kidney of DR-fed mice, as compared to AL-fed ones and also in the T₃-treated mice, compared to untreated ones. These findings suggest that malate-aspartate shuttle enzymes are differentially regulated during DR in mice, in order to adapt to the metabolic need of liver and kidney. T₃ potentially regulates the shuttle enzymes, albeit to a varying degree in the liver and kidney of AL- and DR-fed mice.

Keywords: Malate-aspartate shuttle enzymes, dietary restriction, triiodothyronine (T₃), liver, kidney, mice.

The malate-aspartate shuttle, comprising of mitochondrial and cytosolic aspartate aminotransferase (m- and c-AsAT; EC 2.6.1.1) and malate dehydrogenase (m- and c-MDH; EC 1.1.1.37) is a major pathway for the transport of reducing equivalents (-H or electrons) from cytosol to mitochondria in many animal tissues¹⁻³. The inner mitochondrial membrane is impermeable to NADH, which is formed by glycolysis in the cytoplasm during oxidation of glyceraldehyde-3-phosphate. NADH must be regenerated to NAD⁺ for glycolysis to operate. 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 cycle⁴⁻⁶. The compartmentalization of human c-MDH, together with its isoenzyme m-MDH plays an important role in the aerobic metabolism of the malate-aspartate shuttle

and the citric acid cycle⁷. Thyroid hormone regulates the malate/aspartate and α -glycerophosphate shuttle pathways in cardiac mitochondria, either directly by altering gene expression or indirectly by increasing myocardial workload⁸.

The dietary restriction (DR) i.e., a reduction in caloric intake without malnutrition influences several physiological processes viz., immunological, protein and amino acid metabolism, and also neuro-endocrinological system⁹⁻¹². 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 tumors¹³⁻¹⁶. It may enhance longevity by potentiating the immune responses and lowering the oxidative stress/damage¹⁵⁻¹⁷. It significantly increases gluconeogenesis that concurs with an increased rate of protein turnover during such intervention¹⁸.

Earlier, influence of diet composition as a regulator of hepatic intermediary metabolism has been reported in rats¹⁹. The rats fed with low-protein diet had an elevated plasma concentration of both total and free triiodothyronine (T₃), but the hepatic malate-aspartate shuttle activity was reduced. The energy-restricted diet, however, led to a reduction of T₃ in rats²⁰. Hence, feeding a low-protein and/or energy-restricted

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Abbreviations: AL, *ad libitum*; DR, dietary-restriction or dietary-restricted; T₃, triiodothyronine; c- and m-AsAT, cytosolic and mitochondrial aspartate aminotransferase, respectively; c- and m-MDH, cytosolic and mitochondrial malate dehydrogenase, respectively.

diet had differential effects on the level of plasma T_3 . As the role of malate-aspartate shuttle is very critical in the oxidative metabolism⁶, its possible alteration by dietary control and T_3 treatment may provide an insight into the regulation of this shuttle during such interventions. The present study was aimed at understanding the regulation of malate-aspartate shuttle in the liver and kidney of mice during long-term dietary restriction and T_3 treatment.

Materials and Methods

Animals and chemicals

Swiss albino (Balb/c strain) male mice of 8 weeks, maintained under normal laboratory conditions were fed with a standard pellet diet (Amrut Laboratory, Pune) and water *ad libitum* as per experimental schedule. Dietary-restricted mice were fed on alternate days for a period of 3 months^{21,22}. Animals were sacrificed at the end of a feeding day.

All the chemicals used were of analytical grade and the biochemicals were purchased from Sigma Chemical Co., USA.

T_3 treatment

The effect of T_3 was studied on the activities of shuttle enzymes in the liver and kidney of both AL- and DR-fed mice. Trial experiments were carried out to determine the time- and dose-response of the shuttle enzymes towards T_3 . Maximum response of the enzymes was obtained with a dose of 200 $\mu\text{g}/100$ g body weight after 6 hr of T_3 injection. Both AL- and DR-fed mice were administered intraperitoneally (i.p.) with T_3 dissolved in normal saline having 10% ethanol and sacrificed after 6 hr of the treatment.

Tissue preparation and assay of shuttle enzymes

Mice were sacrificed by cervical dislocation at a fixed time of the day (13:00 hr) and their liver and kidney were taken out, washed in chilled normal saline (0.9% NaCl) and blotted dry with filter paper. The 10% (w/v) homogenates of these tissues were prepared in ice-cold sucrose solution (0.25 M) using a homogenizer fitted with a Teflon pestle. The homogenates were centrifuged at $800 \times g$ for 10 min at 0°C to sediment nuclei. The resulting supernatants were further centrifuged at $14,000 \times g$ 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 10 mM potassium phosphate buffer, pH 7.5/0.25 M sucrose/0.5% Triton X-100, and for the assay of m-MDH in 50 mM potassium phosphate buffer, pH 7.5 containing 0.25 M sucrose. Assays were performed within 3 hr of mitochondrial suspension.

The activities of c- and m-AsAT were measured as described²³, with some modifications^{5,24}. The isoenzymes c- and m-MDH were assayed spectrophotometrically as described²⁵, with certain modifications⁵. The rate of oxidation of NADH was measured at 340 nm using a cuvette of 1.0 cm light path. The activities of both isoenzymes of MDH and AsAT 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 employing the Bradford method²⁶ using bovine serum albumin as standard.

Reconstitution of malate-aspartate shuttle

For reconstitution of the malate-aspartate shuttle, tissues were homogenized in 4 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4/0.25 M mannitol/1 mM EDTA/2 mM MgCl_2 /30 mM 2-mercaptoethanol) and centrifuged at $800 \times g$ for 10 min at 0°C to sediment nuclei. The supernatant was further centrifuged at $14,000 \times g$ for 30 min to sediment mitochondria. The mitochondrial pellet was washed twice, suspended in homogenization buffer and used in reconstitution studies. The post-mitochondrial supernatant was dialyzed for 18 hr at 4°C against 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 2 mM MgCl_2 , and 30 mM 2-mercaptoethanol. The dialyzed cytosol was centrifuged at $14,000 \times g$ for 30 min to remove the traces of particulate materials and the resultant clear supernatant was used in reconstitution.

Reconstitution assay was done as described^{1,27}, with certain modifications³ of our own in the amount of protein taken and in the final assay volume. The reaction mixture (2.5 ml) contained the following: buffer incubation medium (300 mM mannitol/10 mM potassium phosphate buffer, pH 7.4/10 mM Tris-HCl, pH 7.4/10 mM KCl/5 mM MgCl_2 /2 mM ADP/2 mM aspartate), 2 mg cytosolic and 1 mg mitochondrial protein. After setting of the baseline to zero, 50 μl of 7 mM NADH was added to the sample cuvette giving the absorbance of 0.73. The slow steady fall in absorbance was monitored for 2 min and then 50 μl of a solution of 0.2 M each of L-malate and glutamate

was added to both cuvettes. The decrease in absorbance was followed up to 10 min.

Statistical analysis

Data obtained from different sets of experiments were analyzed using Student's t-test. The level of significance ($P < 0.05$) between two sets of data was taken as significant.

Results and Discussion

The body weight of mice during alternate days of feeding for 3 months showed a significant decrease (-38%) in dietary restricted (DR) ($21.3 \text{ g} \pm 1.7$; $n = 7$), as compared to *ad libitum* (AL)-fed mice ($34.14 \text{ g} \pm 2.4$; $n = 7$) (Fig. 1). The weight of the liver and kidney also showed a significant decrease (-19%) during DR, as compared to AL-fed mice (data not shown). These observations confirmed that the mice were indeed subjected to a reduced food intake and are consistent

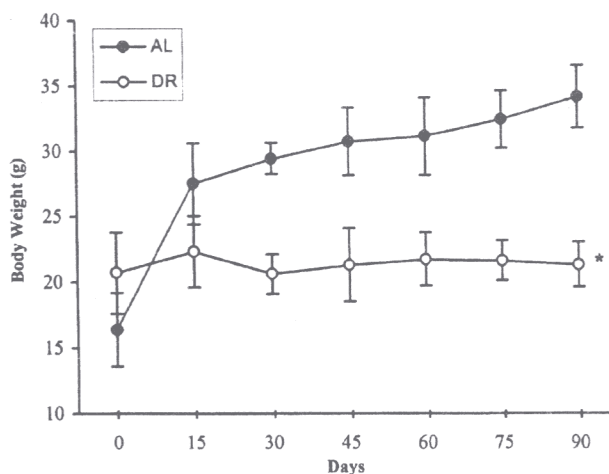


Fig. 1—Body weight of *ad-libitum* (AL) and dietary-restricted (DR) mice [Body weight in both groups was measured at 15 days interval for 90 days. Values are expressed as mean from 7 mice at each point. Bars represent standard deviation. Asterisk (*) represents statistically significant value as compared to AL-fed mice]

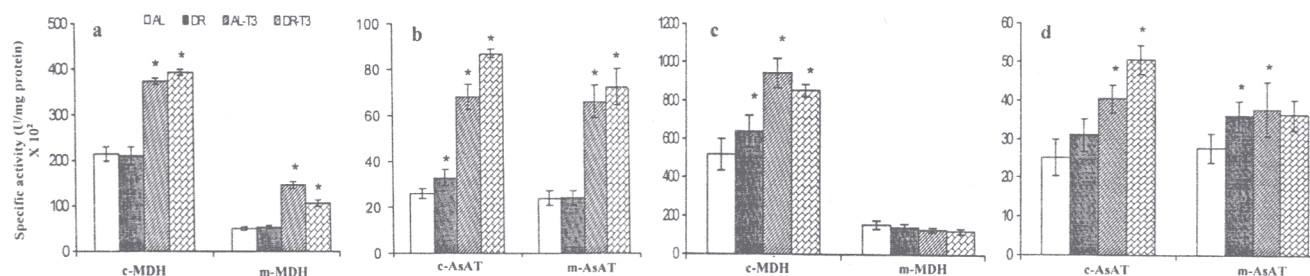


Fig. 2—Activity of c- and m-MDH AsAT in the liver (a and b) and kidney (c and d) of AL and DR mice and also during T₃ treatment to those mice [Values are expressed as mean from 5 mice in each group. Bars represent standard deviation. Asterisks (*) represent statistically significant values as compared to AL-fed mice]

with the earlier report²². Our findings indicate that during DR, c-AsAT activity is significantly increased (26%) in the liver, as compared to AL-fed mice, but there was no change in other isoenzymes of the shuttle (Fig. 2a and b). c-AsAT is known to be involved in gluconeogenesis^{5,28} and its increase does signify more gluconeogenesis in liver during long-term DR. This is in agreement with an earlier report that the dietary calorie restriction in mice leads to an increase in mRNA and/or activity of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase involved in hepatic gluconeogenesis²⁹.

The c-MDH and m-AsAT are increased (24% and 31%, respectively) during DR in kidney (Fig. 2c and d). The increased c-MDH activity may favour more transfer of reducing equivalents to the mitosol by greater production of malate, which is an intermediate substrate of the Krebs cycle. The increase in m-AsAT may lead to more production of aspartate that may help in generating cytosolic oxaloacetate to be utilized by c-MDH during such interventions.

It has been observed in an *in vitro* reconstituted malate-aspartate shuttle that the shuttle activity (as judged by oxidation of NADH) is relatively higher in both liver and kidney during DR (Fig. 3a and d). The observation of the present study is contrary to an earlier report, wherein feeding of low-protein and/or energy-restricted diet led to a reduction in hepatic malate-aspartate shuttle activity in rats²⁰. Such difference could be due to the dietary regimen followed as well as the animal species used in our experimentation.

Keeping in view the metabolic adjustments that take place during DR, the effect of thyroid hormone triiodothyronine (T₃) was studied on the shuttle enzymes in both AL- and DR-fed mice. In liver, the activity of c-MDH is increased in AL-T₃ (+74%) and DR-T₃ (+86%), as compared to their respective

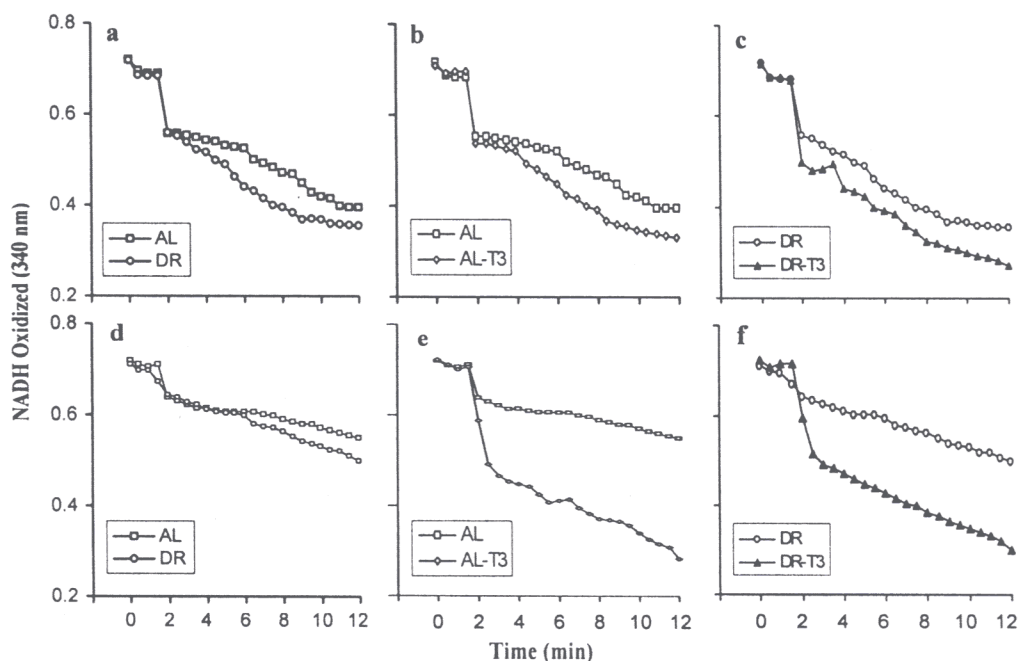


Fig. 3—Oxidation of NADH by reconstituted malate-aspartate shuttle in the liver (a,b,c) and kidney (d,e,f) of mice [(a,d), AL and DR mice; (b,e), AL and AL-treated with T₃; (c,f), DR and DR-treated with T₃. Equal amounts of dialyzed clear cytosol (2 mg protein) and mitochondria (1 mg protein) were used for the reconstitution assay. The lines depict the change in absorbance at 340 nm with the passage of time]

controls in both AL- and DR-fed mice (Fig. 2a), however, there was no significant change in the magnitude of induction in either group. The activity of m-MDH also increased (+190% and 98%) by T₃ treatment in the liver of both the groups, albeit the magnitude of induction was significantly greater in AL-T₃, as compared to DR-T₃ mice.

The activity of c- and m-AsAT in the liver is significantly increased (+162% and +164% and +175% and 201%, respectively) by T₃ treatment in the liver of both AL- and DR-fed mice (Fig. 2b). There was no significant change in the magnitude of induction of c-AsAT activity in either group, however, the magnitude of m-AsAT induction was slightly higher (26%) in DR-T₃ mice, as compared to AL-T₃. Thyroid hormone is known to increase the oxygen consumption of almost all the metabolically active tissues³⁰. Thus, our findings have corroborated⁸ with the fact that during T₃ treatment, the metabolic rate increases, leading to the increased activity of malate-aspartate shuttle enzymes to compensate the energy requirements during such intervention.

In kidney too, the activity of c-MDH is increased (+82% and +33%) by T₃ treatment in both AL- and DR-fed mice, compared to control (Fig. 2c); the magnitude of induction is higher (49%) in AL-T₃ than

DR-T₃. Similarly, the activity of c-AsAT is also increased (61% and 63%) significantly by T₃ treatment in both the groups (Fig. 2d). However, m-MDH showed no significant change during T₃ treatment in either group (Fig. 2c), while m-AsAT showed a significant increase (37%) in AL-T₃, but no change in DR-T₃ mice (Fig. 2d). Earlier, we reported that T₃ significantly increases the activity of both c- and m-MDH in the liver and kidney of chicken⁵. In contrast, T₃ did not induce c-AsAT isoenzyme in the liver and kidney of chicken, except m-AsAT in the liver⁵. These findings corroborate the fact that all four isoenzymes of shuttle are genetically-independent, differing from one another among various tissues of chicken⁵, and apparently show species-specificity in their regulation by hormones³¹.

In order to confirm the differential regulation of malate-aspartate shuttle enzymes by T₃ in the liver and kidney, the shuttle activity was studied in a reconstituted system. Reconstitution of the malate-aspartate shuttle showed a higher activity in the liver and kidney of T₃-treated animals, as compared to respective controls in both groups (Fig. 3b, c, e and f). Thus, the shuttle activity exhibited a similar pattern as that of the expression of enzymatic activities in both the tissues. It reflects a greater transfer of reducing

equivalents in both the groups of animals by T₃ treatment. This is in agreement with the previous findings⁸ that the malate-aspartate shuttle activity is increased in hyperthyroidic rats and is tissue-specific.

From the above findings, it is clear that the malate-aspartate shuttle enzymes are differentially regulated during long-term dietary restriction in the liver and kidney of mice, depending on the metabolic need of the specific tissue. It is consistent with the earlier findings of differential expression of malate-aspartate shuttle in the liver and kidney of mice at different post-natal ages³. As the T₃ is a potent oxidative hormone, it increases the oxygen consumption in most of the tissues to facilitate energy demands and that may increase the shuttle activity to harness the reducing equivalents effectively^{8,31}. This could be accomplished by affecting the malate-aspartate shuttle enzymes during its treatment in both AL- and DR-fed mice. Thus, the regulation of malate-aspartate shuttle enzymes by T₃ may facilitate increased metabolic demands of the liver and kidney to compensate the different tissues requirements during such interventions.

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