Long-term dietary restriction up-regulates activity and expression of renal arginase II in aging mice

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Arginase II is a mitochondrial enzyme that catalyses the hydrolysis of L-arginine into urea and ornithine. It is present in other extra-hepatic tissues that lack urea cycle. Therefore, it is plausible that arginase II has a physiological role other than urea cycle which includes polyamine, proline, glutamate synthesis and regulation of nitric oxide production. The high expression of arginase II in kidney, among extrahepatic tissues, might have an important role associated with kidney functions. The present study is aimed to determine the age-associated alteration in the activity and expression of arginase II in the kidney of mice of different ages. The effect of dietary restriction to modulate the age-dependent changes of arginase II was also studied. Results showed that renal arginase II activity declines significantly with the progression of age ($p<0.01$ and $p<0.001$ in 6- and 18-month-old mice, respectively as compared to 2-month old mice) and is due to the reduction in its protein as well as the mRNA level ($p<0.001$ in both 6- and 18-month-old mice as compared to 2-month-old mice). Long-term dietary restriction for three months has significantly up-regulated arginase II activity and expression level in both 2- and 18-month-old mice ($p<0.01$ and $p<0.001$, respectively as compared to AL group). These findings clearly indicate that the reducing level of arginase II during aging might have an impact on the declining renal functions. This age-dependent down-regulation of arginase II in the kidney can be attenuated by dietary restriction which may help in the maintenance of such functions.

[Majaw T and Sharma R 2017 Long-term dietary restriction up-regulates activity and expression of renal arginase II in aging mice. *J. Biosci.* **42** 275–283]

1. Introduction

Arginase (EC 3.5.3.1) irreversibly hydrolyses L-arginine into L-ornithine and urea. In mammals, its two isoenzymes have been recognized (Cederbaum *et al.* 2004; Pernow and Jung 2013). They differ with respect to subcellular localization, tissue distribution, immunological cross-reactivity and the genes that code them (Jenkinson *et al.* 1996). Arginase type I is predominantly expressed in hepatocytes and its physiological function is well defined in converting highly toxic ammonia into neutral urea that is in turn removed via the urea cycle (Tsang *et al.* 2012). Arginase type II is a mitochondrial, nucleus-encoded protein. Although its physiological function is not well defined (Morris *et al.* 1997), it is broadly expressed in extra-hepatic tissues that lack urea cycle such as kidney and to a lesser extent in other tissues like brain, small intestine, mammary gland and macrophages (Choi *et al.* 2012; Yang and Ming 2014). Since the liver is the only organ to contain all the enzymes of the urea cycle, it is suggested that the presence of arginase II in other tissues has other essential physiological functions besides the urea cycle (Iyer *et al.* 1998). These may comprise of its role in the synthesis of polyamines, glutamate and proline, regulation of nitric oxide synthesis and of arginine homeostasis (Wu and Morris 1998; Levillain *et al.* 2005a). In the kidneys of mouse and human, arginase II is the only isoform expressed suggesting that it may play a key role in renal physiology and homeostasis (Kern et *al*. 2007; Levillain *et al.* 2008). While urea has a vital role in the regulation of urinary concentration (Sands *et al.* 2011), ornithine serves as the substrate for synthesis of polyamines which is important for cell survival (Zahedi *et al.* 2007). The physiological significance of arginase II includes suppressing severity of asthma by dampening inflammatory signal transduction (Xu *et al.* 2016) and

Keywords. Aging; arginase II; dietary restriction; kidney; mice

DOI: 10.1007/s12038-017-9683-8

Published online: 18 April 2017

neuroprotection as mice lacking the protein have aggravated brain injuries following cerebral ischemia and excitotoxicity (Ahmad *et al.* 2016). While some studies indicate that arginase II plays a causal role in macrophage (M1 subset) function whereby it is essentially involved in inflammatory response against infections (Yang and Ming 2014), others indicate its expression to be associated with the development of atherosclerosis and insulin resistance (Ming *et al.* 2012).

Aging entails a progressive decline in proteostasis (Hartl 2016), altered induction of many enzymes (Kanungo 1980; Kaczor *et al.* 2006) and responsiveness of tissues and cells to certain hormonal modulators (Roth and Hess 1982) which is accompanied by reduced cellular, organ and systemic function. Aging kidneys are characterized by declined physiological functions including decreased glomerular filtration, disturbed water and electrolyte balance and reduced urine-concentrating ability (Kielstein *et al.* 2003; Sands 2003; Musso and Oreopoulos 2011). However, the underlying mechanism that causes renal failure remain unclear (Speakman and Mitchell 2011).

To date, dietary restriction (DR) is the only natural and non-pharmacological intervention that slows the aging process and extends both lifespan and health span (Fontana *et al.* 2010; Mitchell *et al.* 2010). DR is a decrease in food intake without causing malnutrition. Various types of DR regimes exist that have different life-extending effects on different types of organisms. Every other/alternate day feeding (24 h fasting and re-feeding) reduces 30-40% of food intake (Qiu *et al.* 2010). Rats and mice subjected to such DR regimen exhibit an increase in their mean lifespan by 83% and 27%, respectively (Mair and Dillin 2008). Although lifespan extension is proportionate to the level of food restriction, more than 60% restriction of food has a negative effect (Speakman and Mitchell 2011). DR has been known to influence various physiological processes such as immunological, neuroendocrinological and protein metabolism (Kapahi 2010; MacDonald *et al.* 2011; Bedard *et al.* 2013). It has been suggested that DR may operate by the regulation of energy metabolism as observed from the correlation between low calorie intake and increased lifespan (Anderson and Weindruch 2010). Studies in rats have shown that as the animal ages, decreased protein turnover is ameliorated by DR, where it promotes metabolic reprogramming indicative of a transcriptional shift that favors energy metabolism and increased protein turnover (Lewis *et al.* 1985; Tavernarakis and Driscoll 2002). It also enhances gluconeogenesis with an increasing pace of protein turnover and eliminates damaged proteins as a result of advancing age (Hagopian *et al.* 2003; Goto and Radak 2013). Transcriptional profiling at the molecular level showed that DR arrests many of the age-related changes and activates expression of several genes that are engaged in energy metabolism (Anderson *et al.* 2009). Regulation of metabolism and the involvement of mitochondria appeared to be a crucial determinant during DR (Anderson *et al.* 2009). These transcriptional alterations are indicative of changes in how energy is generated and the fuel is utilized efficiently (Anderson and Weindruch 2010).

The high levels of expression of arginase II in kidney indicate that it has a physiological role in the regulation and maintenance of its functions and as such, altered levels of arginase II with age may have a contributory role to the decline in kidney functions. Therefore, this study is aimed to determine the level of arginase II in the kidney of mice as a function of age and evaluate the effect of dietary restriction in modulating such age-dependent changes.

2. Material and methods

2.1 *Animal models*

Male albino mice (BALB/c) were used. They were kept in normal laboratory conditions at 25±2°C with equal periods of 12 h light and dark cycle. Mice were fed with standard diet (22.43% protein, 48% carbohydrate, and 4.22% fat) and water was freely accessible. Mice of ages, 2-, 6-, and 18-month-old (n=5–6 in each age group) were used for the age-dependent study. Institutional guidelines on the use of animals were followed during entire experimentation.

2.2 *Dietary restriction procedure*

Mice of ages 2- and 18-month-old were used to study the effect of DR. Each age group was divided into DR group and AL group. The DR group was subjected to alternate days of fasting and re-feeding for a period of three months while water was freely accessible. Age-matched, *ad libitum* fed group (AL) had free access to food and water. All experiments were conducted at the fixed time of the day. Both AL and DR mice (n=5–6 mice in DR and AL groups) were sacrificed (at 11.00 h) by the end of the regimen and were 5- and 21-month-old. Body weight of DR mice was significantly reduced when compared to age-matched controls (AL) indicating that these animals were genuinely subjected to DR (Majaw and Sharma 2015).

2.3 *Chemicals and buffers*

Rabbit anti-Arginase II (H-64: sc-20151) polyclonal antibodies from Santa Cruz Biotechnology, Inc., USA, Goat anti-rabbit IgG-ALP conjugate from Bangalore Genei, India, L-Arginine substrate, diacetyl monoxime, and arsenic acid from Sigma Chemical Co., Dallas, USA. All other chemicals used were of analytical grade.

The following buffers were used: (A) 10 mM potassium phosphate buffer, pH 7.5/0.25 M sucrose/0.5% Triton-X100, (B) TBS buffer (20 mM Tris-HCl pH 7.5/500 mM NaCl), and (C) TTBS (20 mM Tris-HCl pH 7.5/500 mM NaCl/0.05% Tween 20).

2.4 *Tissue preparation and mitochondrial isolation*

To avoid any circadian variations, mice were sacrificed at a fixed time of the day (11.00 h) by cervical dislocation. Kidneys were dissected out, washed in normal saline and blotted dry. A 10% tissue homogenate was prepared in 0.25 M sucrose. The homogenate was centrifuged at 800*g* for 10 min at 0°C to sediment the nuclei. The resulting supernatant was further centrifuged at 14,000*g* for 30 min at 0°C to sediment the mitochondria. The mitochondria obtained were washed thrice and froze at −80°C. The mitochondria were thawed and suspended in solubilizing buffer A. Assay was performed within 3 h of mitochondrial suspension (Goyary and Sharma 2008). Mitochondrial protein concentration was determined by the dye-binding method of Coomassie brilliant blue according to Bradford (1976) using bovine serum albumin as standard.

2.5 *Arginase II activity assay*

The activity of arginase was assayed according to Satoh and Ito (Satoh and Ito 1968) with certain modifications. Briefly, the assay mixture containing 0.1 M Tris–HCl, pH 9.5, 0.85 M L-arginine, and 100 µL of the mitochondrial suspension was incubated at 37°C. The reaction was terminated with acetic acid (87%) after 10 min. To this, 0.073 M $Ba(OH)_2$ and 0.275 M $ZnSO_4$ were added and centrifuged at 3000 rpm for 10 min. One hundred microliters of the supernatant were mixed with diacetyl monoxime (1% in 5% acetic acid) and arsenic acid (100 mg/ml in HCl) and incubated in boiling water for colour development. After 10 min, the mixture was cooled in ice and absorbance was read at 475 nm. The activity of the enzyme is expressed as Unit per mg protein. One unit of enzyme is defined as µmoles of urea produced per minute.

2.6 *Immunoblot analysis*

Arginase II protein level was quantified by immunoblotting using anti-Arginase II antibody (H-64: sc-20151). Sixty micrograms of protein were subjected to 10% sodium dodecyl sulphate–polyacrylamide gels electrophoresis (SDS-PAGE) at constant 40 mA for 60 min and electroblotted to nitrocellulose membrane at constant voltage (100 V) for 1 h at 4°C. It was then incubated in a solution of 5% non-fat dry milk in buffer B for 60 min at room temperature. Blots were then incubated with rabbit anti-Arginase II primary antibodies (1:500) diluted in blocking solution, overnight at room temperature. Following buffer C and B washes, blots were incubated with secondary antibody goat anti-rabbit IgG-ALP conjugate (1:1000), diluted in blocking solution for 3 h. Colour was developed by adding BCIP/NBT substrate. The reaction was stopped by washing the blot with ddH_2O and photographed using HP Scan jet 7400C. The blots were quantified densitometrically using Kodak Digital Science 1D Image Analysis Software, Version 3.0.

2.7 *Coomassie brilliant blue staining (CBB)*

In order to ascertain the equal amount of loads for Western blots, a parallel set was run and the gels obtained after electrophoresis were stained with Coomassie Brilliant Blue (CBB-R250) overnight. The gels were then destained with several changes of the destaining solution. The gels were photograph using HP Scan jet 7400C and quantified densitometrically using Kodak Digital Science 1D Image Analysis Software, Version 3.0. A representative band was selected and used as a loading control.

2.8 *Quantitative real-time PCR*

Total RNA was extracted from kidneys using ice cold TRIsure (BIO-38033) following manufacturer's instruction. Concentration and purity of RNA were determined spectrophotometrically at absorbance 260 and 280 nm. Two micrograms of RNA was reverse transcribed (Thermo scientific, AB-1452/A) and amplified, using SYBR® Select master mix (Applied Biosystem), on a 7500 Fast Real-Time PCR System (Applied Biosystems) using the primers given in table 1. The primers were first standardized and the specificity and efficiency of the primers used were checked by standard curve method. A slope close to −3.32 (near 100% efficiency) was considered highly efficient. Real-time PCR was performed using the primers at a final concentration of 300 nM and 2.5 µL of cDNA template. Melting curve analysis was also performed at the end of the reaction to check the specificity of the products formed. Data were normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative mRNA expression was quantified by the comparative Ct (2^{-Ct}) method (Livak and Schmittgen 2001) and expressed relative to the young group. The agarose gel electrophoresis was also performed after the experiment.

2.9 *Statistical analysis*

Results are presented as mean ± SEM from 4–6 mice in each group. Data were analysed by one-way ANOVA and if

Table 1. Real-time PCR Primers sequences

		Amplicon size
	Arginase II	
Forward	5′-GATTGATCGACTTGGGATCCA-3′	59
Reverse	5′-TGCCAATCAGCCGATCAAA-3′	
	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	
Forward	5′-CAGGTTGTCTCCTGCGACTT-3′	133
Reverse	5′-CCCTGTTGCTGTAGCCGTA-3′	

significant, *post hoc* Tukey's Multiple Comparison Test was performed. The data were analysed using the GraphPad Prism 6.03 with p-value ≤0.05 taken as significant.

3. Results

3.1 *Arginase II activity and expression level in kidney of mice as a function of age*

The normal endogenous activity of renal arginase II shows that young mice (2-month-old) have the highest activity which declines gradually, with the old mice showing the least arginase II activity (figure 1A). As compared to the young mice (2-month) the level of decrease was observed to be 26% in 6-month-old and 44.6% in 18-month-old mice. Western blot analysis confirmed the concomitant decrease in the protein level with age (figure 1B). To further ascertain, if the decrease in protein level was also due to the decreased transcription of mRNA, we performed real-time PCR of the kidney arginase II mRNA. Results showed that arginase II mRNA level also declines significantly from 2- to 6-month-old mice which corroborate with that of the protein level but mRNA was maintained thereafter in 18-month-old mice (figure 2A and B).

3.2 *Effect of DR on renal arginase II expression*

Earlier data from our laboratory have shown that there was a significant decrease (25%) in the body weight of dietary-restricted mice of both young and old ages. It entailed that the mice were in fact subjected to overall DR regimen (Majaw and Sharma 2015; Hadem and Sharma 2016). We observed that dietary restriction up-regulates the arginase II activity in both young (25.79%) and old (22%) mice as compared to the age-matched *ad libitum* (AL) group (figure 3A). The increased arginase activity paralleled with the increased protein (figure 3B) and mRNA expression levels (figure 4A and B) as ascertained by Western blot and real-time PCR, respectively.

4. Discussion

Our findings show that the level of arginase II activity in kidney gradually declines as the mice age. This age-

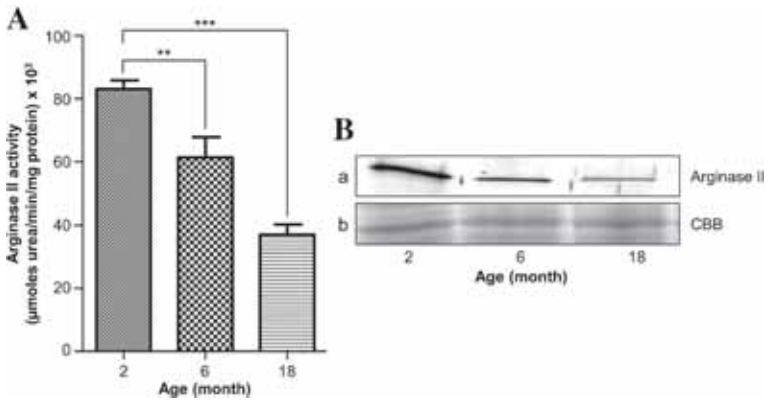

Figure 1. (**A**) Age-dependent change in arginase II activity from the kidney of 2-, 6- and 18-month-old mice. Results are presented as mean±SEM from 4–6 mice. (**B**) (**a**) Western blot analysis of arginase II protein and (**b**) representative band from CBB staining that served as a loading control. Asterisks (** and ***) indicate significant p-values at p≤0.01 and p≤0.001, respectively.

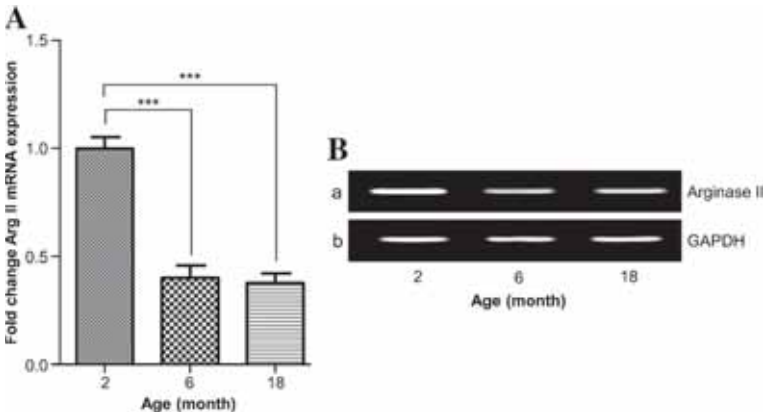

Figure 2. (**A**) Real-time PCR showing the age-dependent change in the level of arginase II mRNA expression from the kidney of 2-, 6- and 18-month-old mice and (**B**) the agarose gel electrophoresis of the PCR products. GAPDH served as an internal control. Results are presented as mean±SEM from 4–6 mice. Asterisks (***) indicate the level of significance ($p \leq 0.001$).

dependent decline of enzyme activity is due to changes in the expression level of the protein. Although arginase II mRNA level also declines between 2- and 6-month-old mice correlating with that of protein level, however, a constant level was maintained thereafter with no changes in the 18-month-old mice. This may happen due to the decrease in translation efficiency and the rate of protein synthesis as the animal ages (Soreq *et al.* 1983; Merry *et al.* 1987). Although the physiological role of arginase II in kidney mitochondria is not well defined, our findings suggest that higher arginase II activity and expression in young mice may be required to support the growth phase of the animal. Since kidneys lack urea cycle, arginase II has roles other than urea synthesis and may be involved in several physiological roles associated with renal functions. The biochemical reaction carried out by arginase II simultaneously decreases arginine concentration and generates ornithine. Ornithine is not the end product of metabolism but plays a pivotal role in two renal metabolic pathways (Levillain *et al.* 2005b). These include synthesis of polyamines by ornithine decarboxylase (ODC) and proline by ornithine aminotransferase (OAT). These biomolecules are essential for the regulation of various cellular processes like cell proliferation, membrane transport and protein synthesis. Reports have shown that inhibition of polyamine synthesis stops cell growth and proliferation (Morrison and Seidel 1995; Igarashi and Kashiwagi 2010). Thus, high arginase II expression in the kidney of young mice may be required to support the enhanced growth of an

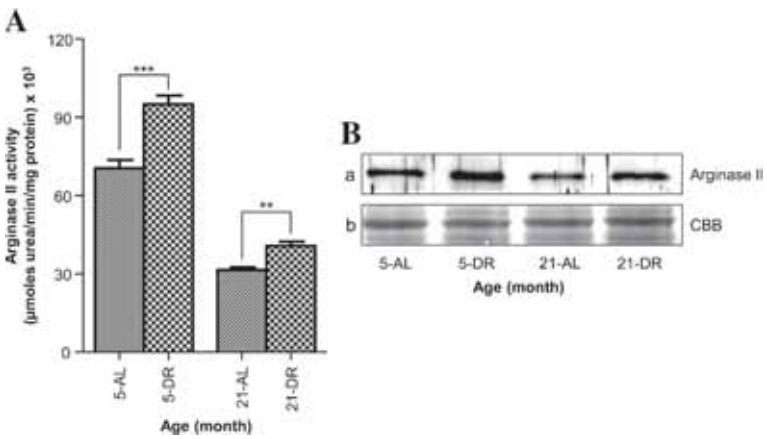

Figure 3. (**A**) Arginase II activity from the kidney of mice subjected to DR. (**B**) Western blot analysis of (**a**) arginase II protein and (**b**) representative band from CBB staining that served as a loading control. Data are expressed as mean±SEM from 4–6 mice. Asterisks (** and ***) indicate significant *p*-values at $p \leq 0.01$ and $p \leq 0.001$, respectively.

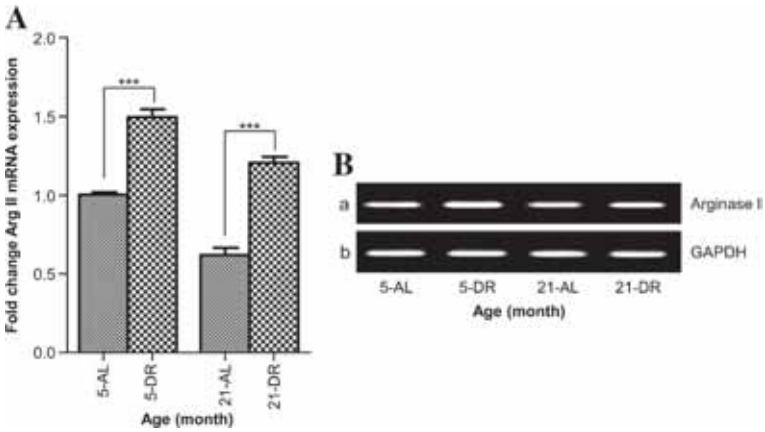

Figure 4. (**A**) Real-time PCR showing the level of arginase II mRNA expression from the kidney of DR mice and (**B**) the agarose gel electrophoresis of the PCR products. Data are presented as mean±SEM from 4–6 mice. Asterisks (***) indicate significant value of $p \leq 0.001$.

organism by providing a precursor for the ongoing/subsequent high rates of protein synthesis during early development. The gradual decrease in the level of arginase II with the advancement of age may be associated with the diminished functions and capability of an organism that results in the gradual cessation of growth and proliferation of cells in aging mice. A similar finding was also observed in an earlier study where the level of arginase II gradually decreases with increasing age in the kidney of albino rats (Patnaik and Patnaik 1989). These findings are consistent with our results thereby supporting the hypothesis that declining arginase II activity with age is associated with decreased functional capability of the kidney as the animal ages and that mitochondrial arginase II activity is linked to kidney functions. Urea, produced by arginase II in the mitochondria, plays a critical role in kidney functions. It serves as an important solute for the development of osmotic gradient in the renal medulla (Bankir *et al.* 1996; Sands 2007), lowers blood pressure in several rat models by acting as simple osmotic diuretics (Iwata *et al.* 2002) and inhibits the Na-K-2Cl cotransporter in the renal tissue (Kaji *et al.* 1997) thereby playing a role in the counter-current exchange system of the nephrons to regulate water and electrolyte balance. The coexistence of arginase II with OAT in the mitochondria indicates that the L-arginine-derived ornithine is a preferred source of glutamate production under physiological conditions channeling the glutamate to enter the tricarboxylic acid cycle for ATP production (Levillain *et al.* 2005b). Therefore, reduced arginase II activity in the kidney may lower urea production which may increase sodium reabsorption contributing to increasing blood pressure and decreasing ATP production as the animal ages. Arginase II is implicated to have a protective role as evidenced by reports indicating that down-regulation of arginase II enhances renal apoptosis while its overexpression prevents apoptosis during exposure to inorganic mercury (Kanda *et al.* 2008). Similarly, recent evidence has also indicated that decreased expression of arginase II in kidney accelerates the renal damage associated with obesity in mouse model (Romero *et al.* 2013). Increasing evidences show that arginase II activity plays a role in vascular functions by regulating the vascular tone through interaction with nitric oxide synthase (NOS). Studies have indicated that in the lungs of rats, arginase II activity and expression level was developmentally regulated with young rats showing the highest expression level. Increasing arginase II activity in the young is associated with regulation of smooth muscle tone in early life (Belik *et al.* 2008).

DR brings about reprogramming of metabolic rate by increasing the expression of genes involved in energy metabolism and increasing biosynthesis and turnover of protein (Tavernarakis and Driscoll 2002; Anderson and Weindruch 2010). Our data indicate that arginase II activity and expression level were up-regulated by dietary restriction. Up-regulation of arginase activity and expression level were observed to be higher in the younger as compared to the older mice. These results indicate that increased arginase II activity by DR is due to the increased arginase II protein and mRNA expression levels. Arginase plays a pivotal role in the metabolic pathway that allows the most favorable utilization of arginine during metabolic demand. The concomitant up-regulation of arginase II is

essential to commensurate with increasing rate of protein turnover during DR by providing ornithine, a precursor for proline and glutamate synthesis. The co-localization of OAT with arginase II in the mitochondria supports the notion that favors the conversion of ornithine to glutamate and proline (Dhanakoti *et al.* 1992; Levillain *et al.* 2004). The function of mitochondrial arginase II is poorly understood, but it can be suggested that increase expression of arginase II in mitochondria may have an impact on the cellular bioenergetics. L-ornithine can be channeled to form glutamate, where through various transamination reactions including the formation of α-ketoglutarate (αKG), may enter the TCA cycle and increase the intermediate flux for greater cycle activity. Studies in lungs of asthma patients indicate that increase arginase II expression is accompanied by increased activity of the electron transport chain in the mitochondria (Xu *et al.* 2016). On the other hand, up-regulation of arginase II by DR may increase the production of urea which might be beneficial in the regulation of water and electrolyte balance thereby contributing to the maintenance of physiological homeostasis during aging. Reports also revealed that increasing arginase II in the mitochondria enhances arginine flux that in turn increases oxidative metabolism, modulates redox balance and suppresses pathological inflammatory events (Xu *et al.* 2016). Hence, from our findings, it can be suggested that increased arginase II with DR has a beneficial effect of increasing mitochondrial bioenergetics that is required for maintenance of cellular homeostasis.

In conclusion, our studies indicate that arginase II activity and expression declines during aging in the kidney of mice and the long-term dietary restriction ameliorates the activity and expression of this enzyme in aging animals. Since kidneys lack urea cycle, it is suggested that the arginase II in kidney may have a contributory role in regulation of most aspects of arginine metabolism during aging and up-regulation of arginase II expression by DR may be important for osmotic regulation and may account for the associated cellular proliferation by diverting L-arginine to form polyamines and proteogenic amino acids required for protein synthesis; one of the ways by which DR attenuates changes in metabolic system during aging (figure 5).

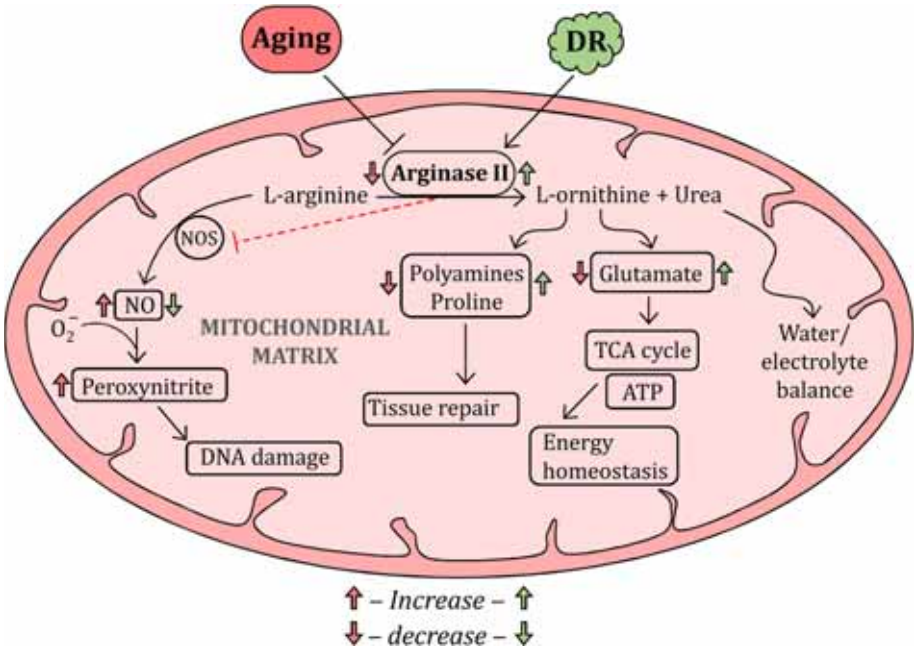

Figure 5. Diagram showing down-regulation of arginase II expression during aging and its up-regulation by DR. Decreased arginase II causes less production of ornithine and urea that may result in the decreased synthesis of polyamines, proline, and glutamate, thereby compromised tissue repair and energy homeostasis and also water and electrolyte balance. The arginine, also a substrate of nitric oxide synthase (NOS), would be available in abundance resulting in excess production of nitric oxide, which on reaction with oxygen radicals, produced peroxynitrite which can induce DNA damage and inhibits superoxide dismutase (SOD). The age-dependent change of renal arginase II can be prevented by DR, shifting towards increased tissue repair, and maintenance of electrolyte balance.

Acknowledgements

We express our appreciation to the Department of Biochemistry, North-Eastern Hill University, Shillong, for assisting with the research facilities under UGC-UPE, SAP and DST-FIST, New Delhi. TM thanks the Council of Scientific and Industrial Research (CSIR), New Delhi, for the financial support as JRF and SRF (F.No: 09/347(0194)/2010-EMR-I).

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MS received 27 November 2016; accepted 28 February 2017

Corresponding editor: Seyed E Hasnain