Tissue-specific expression of receptor-interacting protein in aging mouse

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Abstract Receptor-interacting protein (RIP) is a well-characterized coregulator for nuclear receptors. Here, we report the expression of RIP as two isoforms with molecular weights of 140 kDa and 137 kDa in liver and kidney, but only as one isoform of 140 kDa in lung, adipose tissue, prostate and testis of mice. The levels of both the isoforms decreased in liver and kidney of old mice compared with adult mice. The expression of RIP140 in kidney was relatively lower in old males than females. In contrast, adipose tissue showed remarkably higher levels of RIP140 in old than adult mice of both sexes. Thus, the expression of RIP varied with the type of tissue, sex and age of mice, suggesting differences in its function as a coregulator.

Keywords Receptor-interacting protein · Coregulator · Expression · Aging · Sex · Mouse

Introduction

Receptor-interacting protein (RIP) 140 was discovered as the first transcriptional coregulator for nuclear receptors (NRs). Initially, it was isolated as an

estrogen receptor α partner, though it interacts with many other NRs (Augereau et al. 2006a). It consists of 1,158 amino acids in human and 1,161 amino acids in mouse, with 83% homology between the two sequences. When it is recruited with agonist-bound receptor, it acts as a transcriptional coregulator and inhibits target gene transcription by competition with coactivators (Treuter et al. 1998).

RIP undergoes post-translational modifications like phosphorylation and acetylation. Phosphorylated RIP140 binds to 14-3-3 proteins (Zilliaccus et al. 2001; Darling et al. 2005), which interact directly with a large number of target proteins and alter their activity, localization and protein-protein interactions.

RIP140-null mice are sterile, suggesting its role in fertility (Augereau et al. 2006a). It controls the balance between energy storage and expenditure through the regulation of specific genes involved in energy metabolism (Christian et al. 2005a, b; Powelka et al. 2006). It negatively regulates cellular respiration, citric acid cycle, glycolysis and hexose uptake by silencing the genes involved in these metabolic pathways. It acts as a broad negative regulator of multiple metabolic pathways in adipocytes and might therefore be considered as a putative therapeutic target for metabolic syndromes.

Liver, kidney and adipose tissue are involved in energy metabolism and other important functions which change with age. As the expression of RIP140 is higher in these tissues and it is a key regulator in various pathophysiological processes related to energy

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metabolism, we have measured its levels in the liver, kidney and adipose tissue of adult and old mice. As RIP140 has role in reproduction, we studied the expression of RIP140 in testis and prostate in order to understand its expression during aging.

Materials and methods

Animals

Male and female adult (25 ± 5 weeks) and old (65 ± 5 weeks) mice of AKR strain were used for the study. The average lifespan of these mice under our laboratory conditions is about 75 weeks. They were maintained in a colony at 25°C with an alternating 12-h dark and light schedule and having free access to standard mice feed and drinking water. Experimental protocols were approved by the animal ethical committee of Banaras Hindu University, Varanasi, India. Three mice of each age and sex were used for each experiment. The tissue samples from three mice were pooled. The mice were randomized. Experiments were repeated three times.

Preparation of nuclear extract

Nuclei were purified from the mouse liver, kidney, adipose tissue, brain, heart, lung, prostate, intestine and testis according to Hewish and Burgoyne (1973) with few modifications. The tissue was homogenized separately in solution A, composed of 0.34 M sucrose, 0.2 mM PMSF, 2 mM EDTA, 0.2 mM EGTA, 0.5% Triton X-100 in buffer A (60 mM KCl, 15 mM NaCl, 15 mM Tris-Cl pH 7.4, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β -mercaptoethanol). The homogenate was passed through four layers of cheese cloth and centrifuged for 15 min at 2,500g at 4°C. The pellet was washed with solution A and resuspended in solution A. Then it was layered over two volumes of solution B, composed of 1.8 M sucrose, 0.2 mM PMSF, 2 mM EDTA, 0.2 mM EGTA in buffer A, and centrifuged at 25,000 g for 1 h at 4°C. The pellet was washed with solution C, containing 0.34 M sucrose, 0.2 mM PMSF in buffer A. The pelleted nuclei were resuspended in solution C and stored in 50% glycerol.

The purified nuclei were used to prepare the nuclear extract (NE) according to Dignam et al

(1983) with few modifications. The nuclei were lysed in lysis buffer and the lysate was centrifuged at 25,000 g for 30 min at 4°C. The supernatant fraction was collected and the protein content was estimated by Bradford (1976) method.

Western blotting and detection

Twenty-five microgram of nuclear proteins from liver, kidney, adipose tissue, brain, heart, lung, prostate, intestine and testis of adult male mice were separated by 8% SDS-PAGE and transferred onto PVDF membrane (Amersham Biosciences, USA). The transfer efficiency was checked by Ponceau-S staining. Then the stain was completely removed from the membrane by repeated washing in water. The membrane was blocked with 5% non-fat milk in 1× phosphate buffered saline pH 7.4 (PBS) for 2 h at room temperature. Then it was incubated with anti-human RIP140 monoclonal antibody H-300 (Santa Cruz Biotechnology, USA; 1:1,000 dilution) at 4°C overnight. The blot was washed three times (5 min each) in 1×PBS and incubated with anti-rabbit IgG-HRPO conjugate (Sigma; 1:2,000 dilution) for 2 h at room temperature. Finally, the blot was washed three times (5 min each) in 1×PBS and signals were detected by ECL method (Amersham Biosciences, USA). After detection of RIP140, the blot was stripped in the presence of 62.5 mM Tris-Cl (pH 6.8), 2% SDS and 100 mM β -mercaptoethanol at 50°C for 30 min. The blot was washed in autoclaved triple-distilled water with several changes and then blocked again with 5% non-fat milk and 1×PBS for 2 h at room temperature. The blot was reprobated with anti-actin/tubulin primary antibody (A2668; Sigma; 1:2,000 dilution) and anti-rabbit IgG-HRPO conjugate (Sigma; 1:2,000 dilution). Washing and detection were done as described above.

For aging study, 50 μ g of proteins from liver, kidney, adipose tissue, prostate, and testis of adult and old mice were separated by 8% SDS-PAGE and immunoblotting was performed as described earlier.

Densitometry and statistical analysis

Bands obtained after ECL detection on the X-ray film were scanned and their intensities were quantified using Flurochem software, version 2.0 (Alpha Innotech, USA). The mean and standard error of the mean

Fig. 1 Western blot showing expression level of 140-kDa and 137-kDa RIP in different tissues of mouse. The histogram represents integrated density values of RIP/tubulin $\pm$ SEM from three independent experiments

(SEM) were calculated for the data obtained from three independent sets of experiments after normalization with actin/tubulin. The values were expressed as mean $\pm$ SEM. The level of significance was determined by Student's paired *t*-test and $P < 0.05$ was considered as statistically significant.

Results

Expression of RIP in different tissues

In mouse liver and kidney, we observed two isoforms of RIP with molecular weights of 140 kDa and

Fig. 2 Age- and sex-dependent changes in the RIP level of mouse liver (a) and kidney (b). The histograms represent integrated density values of RIP/actin $\pm$ SEM from three independent experiments. An asterisk indicates significant difference ($P < 0.05$) between two groups: *a** shows significant difference between adult and old male; *b** shows significant difference between adult and old females; *c** shows significant difference between old males and old females

Table 1 Effects of age on RIP expression (A adult, O old)

Protein	Liver		Kidney		Adipose tissue		Prostate	Testis
	Male	Female	Male	Female	Male	Female	Male	Male
140-kDa RIP	A>O	–	A>O	A>O	A<O	A<O	–	–
137-kDa RIP	A>O	–	A>O	A>O	No expression	No expression	No expression	No expression

137 kDa. However, in adipose tissue, brain, heart, lung, prostate and testis, only a single isoform of RIP with molecular weight of 140 kDa was detected. The expression of 140kDa RIP was significantly higher in liver, kidney, brain and prostate compared with other tissues (Fig. 1).

Age-dependent differences in RIP level in liver, kidney, adipose tissue, testis and prostate

To know the effect of age on the expression level of RIP, the intensity of the signal of adult mice was compared with that of old mice. The immunoblot analysis revealed that the expression of the two forms of RIP was significantly lower in old male liver compared with adult male liver (140kDa RIP, $P=0.004$ and 137kDa RIP, $P=0.012$ (Fig. 2a; Table 1).

In kidney, the expression of RIP was significantly higher in adult males than in old males (140kDa RIP, $P=0.044$; 137kDa RIP, $P=0.038$). Similarly, in females, the level of both forms of RIP decreased in old mice compared with adult mice (140-kDa RIP, $P=0.0001$; 137-kDa RIP, $P=0.043$) (Fig. 2b, Table 1).

In adipose tissue, the expression of 140kDa RIP was significantly higher in old than adult mice in both males ($P=0.006$) and females ($P=0.004$) (Fig. 3a, Table 1). To correlate the expression of RIP140 in adipose tissue with fat accumulation, the body weight of adult mice was compared with old mice. The results showed that the body weight of old mice was significantly increased compared with adult mice in both sexes (adult male/old male $P=0.0003$; adult female/old female $P=0.001$) (Fig. 3b).

Fig. 3 a Age- and sex-dependent changes in the RIP protein level of mouse adipose tissue. The histogram represents integrated density value of RIP/tubulin $\pm$ SEM from three independent experiments. An asterisk indicates significant differences ($P<0.05$) between two groups: a^* shows significant difference between adult and old males; b^* shows significant difference

between adult and old females. **b** Age- and sex-dependent changes in the body weight of mice. The histogram represents significant differences ($P<0.05$) between two groups: a^* shows significant difference between adult and old males; b^* shows significant difference between adult and old females

Fig. 4 Age- and sex-dependent changes in the RIP level of mouse prostate (a) and testis (b). The histograms represent integrated density values of RIP/tubulin $\pm$ SEM from three independent experiments

The testis and prostate showed no significant age-dependent difference in the expression of RIP140 (Fig. 4a, b; Table 1).

Sex-dependent differences in liver, kidney, adipose tissue, testis and prostate

Liver and adipose tissue showed no significant sex-dependent differences in the expression of RIP (Figs. 2a and 3a; Table 2). But in kidney, the 140-kDa RIP protein was significantly decreased in old female mice compared with old male mice ($P=0.008$) (Fig. 2b; Table 2).

Discussion

Cellular aging is characterized by a production of oxidatively modified proteins. Cellular proteins are oxidatively modified by free radicals like reactive oxygen species, which damage cellular components like proteins, lipids and nucleic acids, and this leads

to a reduction in their cellular or organ functions, and finally results in the process of aging (Farout and Friguet 2006; Friguet et al. 2000). In our study, the expression of the two forms of RIP decreased with increasing age in liver and kidney. It is likely that the two isoforms of RIP are oxidatively modified with increasing age and so they are not targeted to the nucleus to take part in transcription (Mattson et al. 2002). In old age, expression of RIP140 is decreased due to increased expression of genes such as uncoupling protein 1 (*Ucp1*) or carnitine palmitoyl-transferase 1b (*CPT1b*) which are directly repressed by RIP140 at the transcriptional level (Christian et al. 2005a, b). It has been reported that the metabolic pathways suppressed by RIP140 in adipocytes include glycolysis, triglyceride synthesis, the TCA cycle, fatty acid oxidation, mitochondrial electron transport, and oxidative phosphorylation. Indeed, glucose oxidation and oxygen consumption are significantly increased in adipocytes when RIP140 function is attenuated by siRNA silencing (Powelka et al. 2006). So the decreased expression of RIP140 with increasing age

Table 2 Effect of sex on RIP expression (M male, F female)

Protein	Liver		Kidney		Adipose tissue	
	Adult	Old	Adult	Old	Adult	Old
140-kDa RIP	—	—	—	M>F	—	—
137-kDa RIP	—	—	—	—	No expression	No expression

may increase the fatty acid oxidation, glycolysis, TCA cycle and these metabolic activities generate a large excess of free radicals. Depletion of RIP140 may upregulate the genes that encode enzymes involved in the TCA cycle, glycolysis, fatty acid oxidation, oxidative phosphorylation and mitochondrial biogenesis. These processes generate free radicals, which are the main cause of aging. Our findings show that with increasing age the two forms of RIP are significantly decreased in old male liver compared with that in the adult male. So it may be assumed that RIP is regulated by male sex hormone and its receptor in liver (Augereau et al. 2006a, b). Male sex hormone level decreases with increasing age (Blouin et al. 2005). But our results show that RIP decreased during old age in both male and female kidneys. However, in kidney, RIP140 is significantly decreased in old females compared with old males. It is reported that with increasing age the female sex hormone level decreases. Decline of sex hormone may down-regulate RIP140 level. So it can be predicted that in kidney RIP140 is regulated by male and female sex hormones. In human breast cancer cells, RIP140 mRNA levels are up-regulated by estrogens (Augereau et al. 2006a, b; Cavailles et al. 1994; Kerley et al. 2001). In our study, we noticed that in the kidney of adult females, RIP140 was significantly increased. It can be assumed that it is due to increase in levels of female sex hormone, which regulates the expression of RIP140 and it is a well-known coregulator for ER α (Cavaillès et al. 1994). The 140-kDa RIP is an acetylated form (Huq and Wei 2005). Here, we report the 137-kDa RIP, an isoform which is expressed only in liver and kidney, indicating its specific role in metabolism. Phosphorylation of RIP140 participates in the regulation of its biological activity. Decreased phosphorylation of RIP140 during old age may decrease its activity (Gupta et al. 2005).

In contrast to liver and kidney, we also noticed that in adipose tissue, expression of RIP140 was significantly increased in old mice. Leonardsson et al. (2004) previously reported that RIP140 is expressed at greater levels in adipose tissue. Ucp1 is a member of the mitochondrial carrier family of proteins that, upon activation, uncouples respiration from ATP synthesis, resulting in an increased metabolic rate and the release of chemical energy in the form of heat. The expression of Ucp1 correlates with protection from obesity in adipose tissue. RIP140 directly targets

a characterized enhancer element in the *Ucp1* promoter, which is known to bind NRs. However, the absence of RIP140 in adipose tissue not only allows increased expression of certain genes but also causes decreased expression of other genes such as *Ucp1* (Parker et al. 2006). Our finding suggested that increased expression of RIP140 in old mice decreased the Ucp1 expression and increased fat consumption with the significant gain of weight in old mice compared with adults. RIP140-null mice have a reduced body weight and body fat content. RIP140-null mice are lean because they fail to store triglycerides and, strikingly, are resistant to high-fat diet-induced obesity, indicating that alternative mechanisms are involved in the dissipation of excess fuel (Leonardsson et al. 2004).

RIP140 is expressed in epididymal epithelial cells, in the prostate and the testis (Steel et al. 2005). However, no age-specific changes were noticed in the prostate and testis, indicating a role of RIP140 in reproduction or inactive aggregation of RIP140 in old mice. In prostate cancer cells, the RIP140 mRNA steady-state levels are increased by androgens (Carascossa et al. 2006).

Nuclear receptor coregulator, RIP140 regulates the transcription of metabolic gene networks. Impaired expression of this cofactor or alteration in its function is a cause of different metabolic disorders (White et al. 2008). In a nutshell, we can say that study on expression of RIP in aging mouse liver, kidney and adipose tissue may provide an opportunity for pharmacological intervention in the treatment of obesity, diabetes and other age-related disorders. Further on the 137-kDa RIP in liver and kidney will reveal some new findings for age-related metabolic diseases.

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