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Tissue-specific decline in the orphan nuclear estrogen-related receptor alpha and its target medium-chain acyl-CoA dehydrogenase levels in aged female mice

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ABSTRACT

The estrogen-related receptor alpha (ERRα) is a gene regulator that modulates target genes controlling metabolic and various functions and actively promotes energy homeostasis in animals. Since aging in animals is characterized by deregulation in energy homeostasis, whether ERRα level undergoes modulation during aging was investigated. Western analysis demonstrated positive ERRα expression in various tissues, with the heart expressing the highest ERRα level. The level of ERRα in old (24 months) mice's heart, kidney and skeletal muscle was significantly reduced (by 46.50%, 38.40% and 30.30%, respectively) compared to young (3 months) mice. Moreover, ERRα level was unchanged in the small intestine, liver, lung, uterus and brain of old mice as compared to young animal. The level of ERRα-inducible enzyme, medium-chain acyl-CoA dehydrogenase (MCAD) was also reduced in the heart (by 35%), kidney (by 40%) and skeletal muscle (by 25%), including liver (by 30%) in old mice, but not in other tissues. The unchanged ERRα level in these tissues may be beneficial and help maintain tissue energy balance and other functions. However, decreased cardiac, renal and muscle ERRα along with MCAD level in the old animal might disturb normal nutrient-sensing pathways which may have a negative effect on energy generation and homeostasis.

KEYWORDS

Aging;
ERRα;
Metabolism;
Mice;
Orphan nuclear receptors.

INTRODUCTION

The estrogen-related receptor alpha (ERRα; NR3B1) is an orphan receptor of the ERR (NR3B) subfamily of orphan nuclear receptor (ONR) group that belongs to the larger family of receptors called the nuclear receptor superfamily[1]. The other members of the NR3B subfamily include ERRβ (NR3B2) and ERRγ (NR3B3)[2,3]. ERRα in expressing cells are nuclear localized and upon sensing appropriate signal(s) bind to specific DNA sequence in the promote region to modulate target gene expression[4,5]. Since physiological ligand for ERRα is unknown, it is thought to be constitutively active however, largely dependent on its functional in-

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teraction with PPAR gamma co-activator 1α (PGC-1α) or PGC-1β for optimal gene activation[6,7]. ERRα also interact with co-repressors such as RIP140 leading to target gene repression[8]. Hence, ERRα target genes may be up-regulated or down-regulated through specific co-activator or co-repressor interaction, providing a robust regulatory system.

ERRα regulates cellular energy metabolism and maintain energy balance[5,9]. ERRα controls energy balance and other functions that has a metabolic basis, especially in organs with high energy demand and metabolic activities[10-12]. ERRα regulates the expression of various metabolic genes that participate in fatty acid β-oxidation (FAO), gluconeogenesis, oxidative phosphorylation (OXPHOS) etc.[12-14]. The expression of the ERRα gene (*Esrra*) is tightly regulated which determine its cellular protein level. Caloric restriction, cold exposure and physical exercise in animals have been shown to up-regulate ERRα expression that may help maintain energy homeostasis through adaptive mechanisms[15-17].

Aging affects a number of biochemical and molecular events which may alter homeostatic balances in animals[18,19]. Aging is also characterized by changes in the expression of multitude of genes, many of which codes for transcriptional factors. Aging at the biochemical level is a metabolic process and the loss of metabolic regulation is accompanied with aging. Aging in mice has been earlier shown to reduce the expression level of glucocorticoid receptors (GRs) in the liver and kidney[18]. In the aged mouse cerebral cortex, estrogen receptor (ER) α and β transcript level is markedly reduced[20]. This decline in receptor level may compromise metabolic and other cellular functions and may drive aging in animals.

Aging leads to metabolic changes and imbalance in energy homeostasis in animals[19,21]. However, the effect of aging on cellular ERRα level is unknown in animals. Modulation of ERRα level during aging may have a significant impact on energy balance and other cellular functions. Aging is also characterized by progressive changes in cellular functions[22]. As cellular functions and energy homeostasis is closely linked, any shift in metabolic homeostasis may negatively affect animals physiology[23]. Hence, ERRα may have a pivotal role in maintaining energy homeostasis. Changes in ERRα level may affect cellular responsiveness to extra cellular signals, thereby altering metabolic activities and energy homeostasis. Since aging leads to decrease in energy homeostasis, it is hypothesized that ERRα level may be modulated during aging. Hence, this study was undertaken to find out whether ERRα level is modulated during aging in mice. Results show tissue-specific decrease in ERRα level. Moreover, the level of ERRα-inducible enzyme medium-chain acyl-CoA dehydrogenase (MCAD) was reduced in tissues where ERRα level down-regulation was also observed in the old animal.

EXPERIMENTAL

Animals and chemicals

Swiss albino (Balb/c) female mice of two different age groups, young (3-months) and old (24-months) were used in the study. Mice were maintained in an animal house at 22°C ± 2°C and subjected to 12 hr light/12 hr photoperiod and fed with standard food pellet (Amrut, India). Animals were maintained and sacrificed in accordance with the guidelines of Indian National Science Academy (INSA guidelines for care and use of animals in scientific research, 2000), New Delhi and approval by the IEC.

Tissue isolation

Animals were sacrificed by cervical dislocation (method approved by INSA). Tissues were then quickly excised and removed, rinsed with chilled normal saline (0.9 % sodium chloride), blotted dry and minced. Tissues included brain, liver, kidney, small intestine, skeletal muscle, lung, uterus and heart. Tissues were then stored in an ultrafreezer at -80°C until future use.

Preparation of whole cell extracts

Whole cell extract was prepared in RIPA buffer containing 50 mM Tris-HCl (*p*H 8.0), 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS[17]. Briefly, thawed tissues were homogenized (5% w/v) in chilled RIPA lysis buffer containing protease inhibitors phenylmethyl sulfonyl fluoride (1mM) and sodium fluoride (5mM). Tissue homogenates were then agitated for 2 hrs at 4°C in a shaker for protein extraction and then centrifuged at 12,000 *rpm* for 20 min at 4°C in a centrifuge. The clear supernatant (whole cell extract) obtained was used for SDS-PAGE. Total protein in whole cell extract was

determined using the dye-binding method of Bradford[24].

SDS-PAGE and western blotting

Aliquots of whole cell extracts were heated at 100°C for 5 min with sample loading buffer (Genei, India). Denatured protein samples (40 µg protein/lane) were resolved in 10% SDS-PAGE in a mini gel electrophoresis set up (Cat no. 106724, Genei, India)[17]. The resolved proteins were then electro-blotted onto nitrocellulose membrane (Axiva, India) in a mini electrotransfer system (Cat no. 106819, Genei). The membrane was then blocked with 5% non-fat dry milk (Sagar, India) in tris buffer saline Tween-20 (TBST) for 1 hr at room temperature (RT) and probed with a primary rabbit polyclonal to ERRα antibody (ab16363, Abcam, UK) at 1:500 dilution in TBST overnight at 4°C. Furthermore, the blot was then probed with a secondary goat anti-rabbit IgG-HRP antibody (Cat no. 105499, Genei) at 1:5000 dilutions in TBST for 1 hr at RT. The blot was then incubated with a solution containing TMB-H_2O_2 as substrate for HRP (Cat no. 106037, Genei) to reveal immunoreactive bands. The immunoreactive signals were scanned by densitometry and photographed.

For MCAD level detection by western, similar approach was employed, except that a primary rabbit polyclonal to MCAD antibody (sc-98926, Santa-Cruz Biotech, USA) at 1:500 dilutions was used. Normalization of ERRα and MCAD bands was done using GAPDH as a loading control. Briefly, the original blot was stripped of antibodies and treated with a primary rabbit polyclonal antibody to GAPDH (ab36840, Abcam) at 1:5000 dilutions and then with goat anti-rabbit IgG-HRP antibody as described before[17]. Experiments were carried out separately with six (n = 6) independent animals from each age group.

Statistical analyses

Data obtained from the experiments were analyzed using Student's *t* test. A *P* value of less than 0.05 was taken as statistically significant for the study.

RESULTS

Tissue levels of ERRα

Western blotting approach was employed to determine the expression of ERRα in various tissues. Positive immunoreactive band of ERRα, which was expressed at a size of ~53 kDa, were detected in all tissues utilized, although at varying levels (Figure 1). A relatively high level of ERRα was detected in the heart, kidney, skeletal muscle and small intestine with a mean level of 3.58 ± 0.40, 2.79 ± 0.31, 1.99 ± 0.21 and 1.25 ± 0.16, respectively. However, ERRα level in the uterus and brain was lower at 0.89 ± 0.16 and 0.78 ± 0.12, respectively. On the other hand, hepatic and lung ERRα level were low and quite similar at 0.60 ± 0.08 and 0.64 ± 0.09, respectively. Interestingly, the cardiac ERRα level was the highest detected. The modest level of ERRα observed in the heart, kidney, skeletal muscle and small intestine is understood as these tissues possess high oxidative metabolism and energy demand.

Aging and ERRα level

The level of ERRα in different tissues of old mice was investigated. Tissues from young mice were also included in the study to provide age-related correlations. Whole cell extracts obtained from different tissues of old and young mice were subjected to SDS-PAGE followed by western blotting in order to find out whether the level of ERRα during aging is modulated. The ERRα protein level was significantly down-regulated in the heart (by 46.50 ± 5.20 %), kidney (by 38.40 ± 4.40 %) and skeletal muscle (30.30 ± 3.60 %) of old animal compared to the young ones as shown in figure 2. However, no significant change in ERRα level from the small intestine, uterus, brain, lung and liver of aged mice was detected compared to the young animal. The reduced level of ERRα in the aged animal shows tissue-specificity. Interestingly, cardiac ERRα level from old mice showed the highest decrease, followed by the modest decline in renal and skeletal muscle ERRα.

Aging and MCAD level

To find out whether this decrease in ERRα levels in the old tissues had an impact on the level of one of its target enzyme was undertaken. MCAD and its gene *Acadm* is a well-known ERRα-responsive target that participates in FAO pathway in a number of tissues[25,26]. It was hypothesized that reduction in ERRα level might modulate level of MCAD in these tissues from old mice. Results obtained showed significant decrease in cardiac (by 35%), renal (by 40%) and muscle (by 25%) MCAD levels in old mice compared to young (Figure

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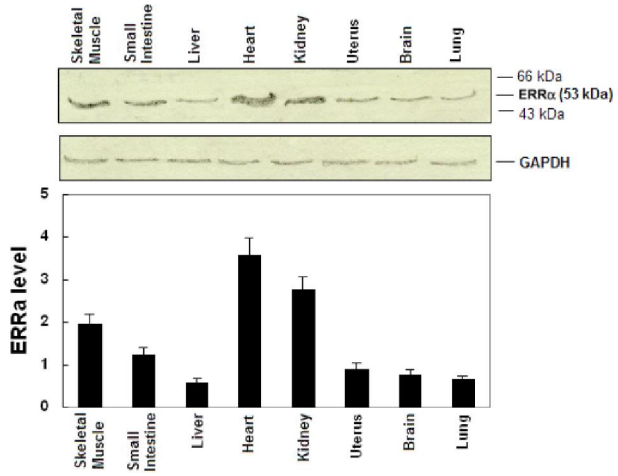

Figure 1 : Western analysis of mouse ERRα level in different mice tissues using primary antibody specific to ERRα (upper panel). 40 µg/lane of total protein in whole cell extracts was subjected to 10% SDS-PAGE and the resolved proteins were transferred onto nitrocellulose membrane for western blotting. Details are provided in the Experimental section. Normalization of ERRα immunoreactive signal was performed using GAPDH as a loading control. Comparison of ERRα levels (Mean ± SD) between various mice tissues is presented as bar graph obtained through densitometric scanning of the bands in the blot (lower panel). Experiments were performed with six independent animals (n = 6). The blot and graph shown is a representative of separate independent analyses on animals

3). Interestingly, MCAD level in the liver was also reduced (30%) in the old animal, a tissue where ERRα level remained the same compared to young ones.

DISCUSSION

ERRα is expressed in majority of the tissues in animals[27]. The detection of modest level of mouse ERRα in the heart, kidney, skeletal muscle and small intestine corroborates with earlier finding, wherein it is showed that tissues that preferentially utilize oxidative metabolism to generate energy express elevated levels of ERRα[28,29]. The high level of ERRα in these oxidative and metabolically active tissues are needed as they principally use FAO and OXPHOS to generate energy. Elevated level of cardiac ERRα expression is necessary, as it primarily utilizes FAO for cellular ATP production by up-regulating enzymes involved in FAO pathway and OXPHOS. Moderately elevated level of ERRα observed in oxidative tissues such as the kidneys, skeletal muscle and small intestine could be due to predominant utilization of fatty acids as energy substrate. Additionally, lower level of ERRα detected in the uterus, brain, lung and liver indicate a comparatively lower preference to FAO for energy production. Brain and liver principally utilize glucose and α-ketoacids respectively, towards ATP production and hence probably express low levels of ERRα. Small intestine also utilizes fatty acids to a large extent to generate energy. However, ERRα level was found to be comparatively lower compared to the heart, kidney and skeletal muscle. This could be due to use of other energy substrates, in addition to fatty acids. Taken together, the level of ERRα were ubiquitous in the tissues used in the study, though at varying levels, with organs with high FAO, OXPHOS and mitochondrial metabolism expressing elevated levels of ERRα.

Aging is associated with a general decline in cellular and physiological processes, which may arise due to fall in energy metabolism and gene expression profiles in animals[18,19]. ERRα has vital functions in controlling oxidative energy metabolism and tissue physiology, which it achieves through regulating target gene transcription[5,12]. One of the factors that ensures precise regulation of such functions is the level of the ERRα itself in expressing cells. Decrease of ERRα level may reduce energy generation and increase imbalance in energy homeostasis, leading to failure of adaptive mechanisms. There is no published report of ERRα level during aging in animals. The significant decrease in ERRα level in the heart, kidney and skeletal muscle from old animal might be as a result of complications of aging. The onset of aging is thought to be due to changes not only at the tissue and cellular level, buy also at the molecular level. Senescence in animals may begin and progress due to decline in the efficiency of gene expression[30]. Previous studies have also shown decline in the level of steroid hormone receptors such as the GRs, ERα and ERβ transcripts in aged rodents[18,20]. Hence, the reduction of ERRα level in the heart, kidney and skeletal muscle in aged mice may lead to fall in efficiency of target gene expression. Furthermore, it is known that ERRα in expressing cells control its own expression by regulating the *Esrra* in functional interaction with ERRγ[31]. Decline in ERRα level during aging may have a negative effect on *Esrra* expression and its product.

The highest decline in cardiac ERRα level compared to other tissues in the old animal may have a significant impact on heart bioenergetics and physiology. Many of

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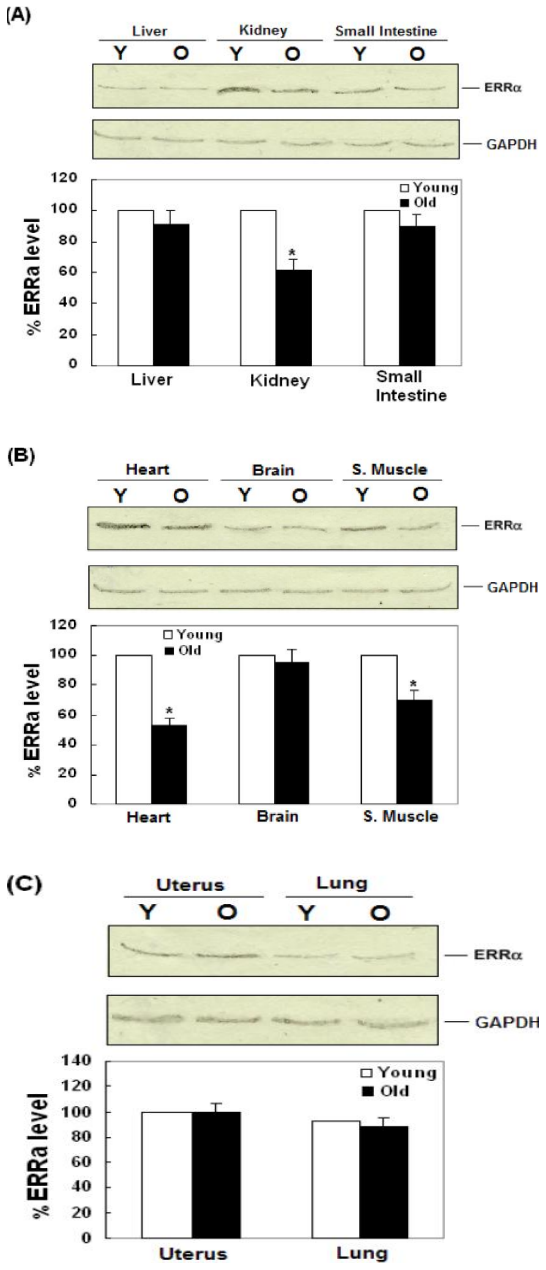

Figure 2 : ERRα level in (A) liver, kidney and small intestine (B) heart, brain and skeletal muscle (C) uterus and lung from young (Y) (3 months) and old (O) (24 months) female mice using primary antibody specific to ERRα (upper panels) by western blotting. Whole cell extracts from the tissues were prepared in RIPA buffer and a total of 40 µg/lane of protein was loaded onto 10% SDS-PAGE and the resolved proteins were transferred onto nitrocellulose membrane for western analyses. Comparison of ERRα level in various tissues from young and old mice is presented as % ERRα level ± SD and indicated as bar graphs (lower panels). ERRα level in young mice tissues were set as 100%. Experiments were done separately with six independent animals from each group. The blot and graph shown is a representative of separate independent analysis on animals. *Significant (P < 0.05) compared to young mice

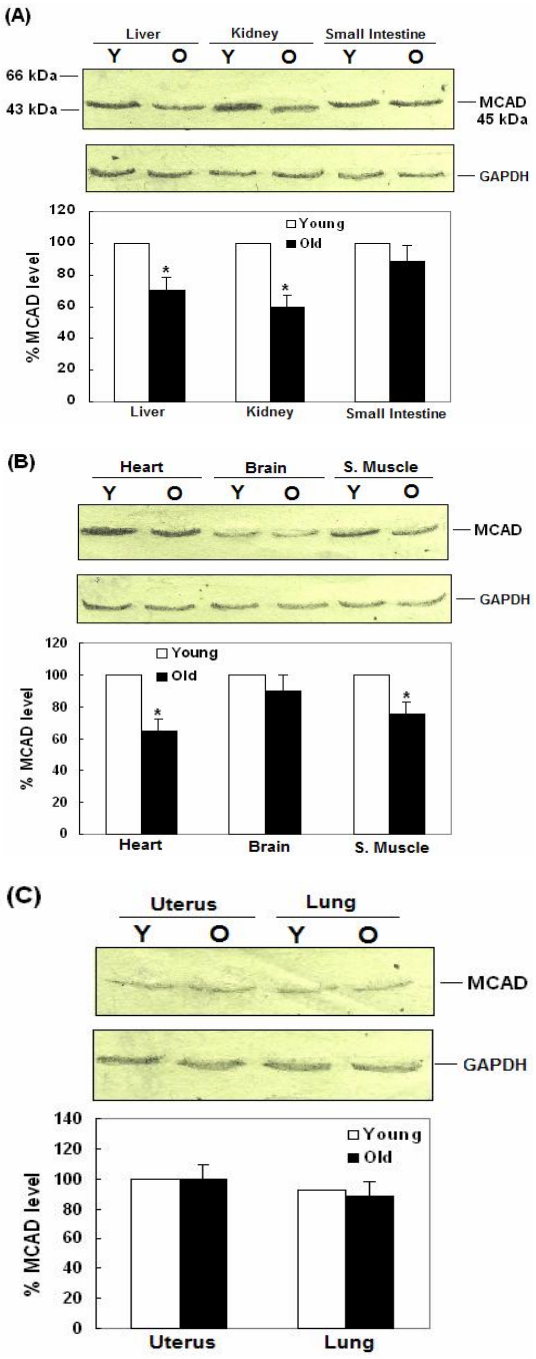

Figure 3 : MCAD level in (A) liver, kidney and small intestine (B) heart, brain and skeletal muscle (C) uterus and lung from young (Y) (3 months) and old (O) (24 months) female mice using primary antibody specific to MCAD protein (upper panels) by western blotting. Comparison of MCAD level in various tissues from young and old mice is presented as % MCAD level ± SD and indicated as bar graphs (lower panels). MCAD levels in young mice tissues were set as 100%. Experiments were done separately with six independent animals from each group. *Significant (P < 0.05) compared to young mice. The blot and graph shown is a representative of separate analyses performed on independent animals

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the genes that participate in energy production and metabolism are in fact target of ERRα[4,5]. These genes may themselves be down-regulated due to low cardiac ERRα level. As heart consumes large amount of ATP and relies heavily on its production from FAO, this fall in ERRα level may reduce FAO rate in the aged heart, compromising energy generation and cardiac functions. Interestingly, the decreased cardiac MCAD level may be as a result of reduced level of cardiac ERRα since *Acadm* is a highly ERRα-responsive target gene. MCAD is a crucial enzyme in FAO pathway and its level is high in oxidative tissues such as heart and which in turn dictate the rate of tissue FAO[32,33]. The reduced level of cardiac MCAD in old mice perhaps indicate an important cellular/physiologic consequence as a result of ERRα down-regulation in the heart which may lead to declined rate of FAO and energy generation. In fact with aging changes in the cardiovascular system have been reported which may lead to alterations in cardiac energy metabolism and physiology such as deterioration in arterial stiffness and elasticity[34]. The fall in cardiac ERRα and MCAD levels during aging may impair adaptive energy metabolism upon exposure to metabolic and other stresses, thereby compromising animal adaptability.

The kidneys expresses high level of ERRα however, during aging the decrease in ERRα level observed may induce renal metabolic reprogramming. Kidneys have critical role in waste excretion, maintenance of body salt balance and so on. These functions are highly energy-dependent on FAO and OXPHOS to generate ATP. Aging in rats is known to be associated with decline in renal functions, being attributed to several factors including decline in urine production, reduction in salt and so on[35]. This fall in renal normal functions may be due to several factors, including decrease in renal ERRα level. Recent study have also demonstrated that ERRα regulate genes for ion channels involved in renal Na^+ and K^+ transport and the renin-angiotensin system[36]. Kidneys are highly oxidative organs and generate much energy through FAO to compensate energy demands. Hence, MCAD level in kidney is also high that perhaps aids in high rate of FAO and ATP production[32,33]. Substantial fall in renal MCAD level observed in old animal could be attributed to decreased renal ERRα level, probably as a result of decreased *Acadm* expression. Thus, the similar per cent decrease observed in renal ERRα and MCAD level during aging may have a substantial negative impact on renal metabolic activities and physiology.

The skeletal muscle utilizes FAO for ATP production needed for muscle contraction. Aging in animals and human is known to cause progressive decline in muscle mass and strength[37]. Amongst many factors, the loss of mitochondrial functions has also been attributed to cause muscle aging as a result of decreased energy generation and increased reactive oxygen species (ROS) production[38]. The decline in ERRα level in aged mice may have a negative effect on FAO and OXPHOS. Decline in OXPHOS efficiency may significantly reduce ATP generation and accelerate ROS generation which may cause mitochondrial dysfunction seen during aging. Additionally, ERRα in association with PGC-1α also control mitochondrial biogenesis[39] and hence lesser level of ERRα in old animal may compromise cellular mitochondrial density. Importantly, MCAD level was also concomitantly reduced in the old muscle, which may be as a result of reduced ERRα level in old animal. This may perhaps bring down the rate of muscle FAO and subsequently ATP level in the old animal. Interestingly, the decrease in hepatic MCAD level observed in old animal in spite of similar ERRα levels in young and old mice could be due to existence of other tissue-specific factor(s) which may also control MCAD level in hepatocytes indicating that ERRα may not be the sole regulator of hepatic *Acadm* activity.

The unchanged ERRα level in the small intestine, uterus, lung, liver and brain of old mice is interesting and shows tissue-specificity of ERRα level during aging. Maintenance of ERRα level in the intestine, uterus, lung, brain and liver of old animal similar to that of young ones may be an adaptation strategy to counter metabolic and other physiological changes in these tissues. Failure of aging to cause any significant change in ERRα expression in these tissues may be due to the existence of tissue-specific factors (such as specific transcriptional factors or co-activators) that prevent decrease in ERRα level or could be as a result of mechanisms currently unknown. In conclusion, this study shows that aging in mice cause significant decrease in ERRα expression in the heart, kidney and skeletal muscle with concomitant reduction in MCAD level as well. This decrease in ERRα level accompanied with MCAD level in these tissues may compromise energy homeostasis and adap-

tive energy metabolism during aging.

ABBREVIATIONS

ERRα: Estrogen-related receptor alpha
FAO: Fatty acid β-oxidation
ONR: Orphan nuclear receptor
OXPHOS: Oxidative phosphorylation
MCAD: Medium-chain acyl-CoA dehydrogenase

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