Expression Analysis of the Key Factors Involved in Mitochondrial Dynamics During Aging in Context with Insulin Signaling

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***Abstract*: Ageing, the process of growing old, is defined as the gradual biological impairment of normal function, probably as a result of changes made to cells and structural components. These changes would consequently have a direct impact on the functional ability of organs, biological systems and ultimately the organism as a whole. While abnormal aging is the rapid deterioration in function due to a pathological process such as a disease like dementia, stroke or acquired brain injury. Several theories given to explain aging and age related disorder but no one explain satisfactorily except free radical theory. The importance of aging research is to understand the age related disorder and to improve the quality of lifespan. As we know mitochondria is the main site for the production Reactive oxygen species (ROS) molecule as a by-product of oxidative phosphorylation. Due to progressive accumulation of ROS molecule mitochondria is more prone to damage because of close proximity. Accumulation of these damages leads cellular dysfunction and age related disorder. As we know insulin is the main hormone in the growth and proliferation during early development, it also helps in mitochondrial biogenesis by activating mTOR and PGC 1 α. Later this acts in negative way by inhibiting autophagy, decreases lifespan and cause abnormal aging. There are more evidences showing that increase in insulin signalling reduces lifespan but it is not clear that up to what extent this reduction increases the lifespan. The observed insignificant changes in the autophagy markers in young and aged brain show that these signaling pathways are differentially regulated in the AKT – mTORC1 level irrespective of the serum insulin levels.**

Keywords: Mitochondrial dynamics, autophagy, neurodegeneration

I. INTRODUCTION

"Aging is the result of the progressive accumulation of deleterious changes that reduce an organism's ability to resist stress causing a decrease in survival possibilities" [1].Thus, aging has four characteristics: a- progressive, b- endogenous, c- universal, and d- deleterious. While abnormal aging is the rapid deterioration in function due to a pathological process such as a disease like dementia, stroke or acquired brain injury. The importance of aging research is to understand the age related disorder and to improve the quality of lifespan. There are several theories of aging but the most excepted one is "Mitochondrial free radical theory" which explains better than others.

This theory is given by Denham Harman. During cellular respiration Reactive Oxygen Species (ROS) molecule is produced as by-product. Due to close proximity with these ROS molecule mitochondria is more prone to damage. Studies in several species reveal a wide spectrum of alterations in mitochondria and mitochondrial DNA (mtDNA) with aging, including (1) increased disorganization of mitochondrial structure, (2) decline in mitochondrial oxidative phosphorylation (OXPHOS) function, (3) accumulation of mtDNA mutation, (4) increased mitochondrial production of reactive oxygen species (ROS) and (5) increased extent of oxidative damage to DNA, proteins, and lipids [2].

Cells have a variety of defenses against the harmful effects of ROS. These include two enzymes i.e superoxide dismutase (SOD) and catalase as well as several small molecules that are antioxidants,such as Alpha-tocopherol (vitamin E),Vitamin C, Uric acid.

Although the body in general has developed several defense mechanisms to counteract oxidative stress, the brain appears to be more susceptible to this damage than any other organ. Although the brain comprises only 2% of the total body weight, it is especially prone to oxidative stress as it consumes about 20% of the resting total body oxygen. Moreover, being postmitotic, neurons in the brain once damaged may be permanently dysfunctional. Because mitochondria is more prone to oxidative damage and these damage is removed by a cellular process called Autophagy in which portions of cytoplasm are sequestered within double membrane cytosolic vesicles termed autophagosomes. The autophagosome cargo is delivered to the lysosome, broken down, and the resulting amino acids recycled after release back into the cytosol. Autophagy occurs in all eukaryotes and can be up-regulated in response to various nutrient limitations. Under these conditions, autophagy may become essential for viability. In addition, autophagy plays a role in certain diseases, acting to prevent some types of neurodegeneration,Type 2 diabetes**,** etc, and in the elimination of invading pathogens. Autophagy is responsible for maintaining cellular health by removing long-

lived and misfolded proteins, damaged and functionally redundant intracellular components [3]. Due to the progressive production of ROS, a small portion of mitochondrial structure tends to be damaged, in this situation ATP generation is compensated by mitochondrial fusion but after prolonged functioning there is elevated damage in mitochondria. These extensively damaged mitochondrial portions undergo fission which favors their engulfment into autophagosomes. Loss of autophagy and/or mitochondrial dynamics leads to condition wherein the ATP requirement is not met in brain cells, hence leading to brain cell dysfunction. Loss of autophagy and/or mitochondrial dynamics leads to damage accumulation in brain, hence leading to brain related disorder. Autophagy is inhibited by the insulin-amino acid-mTOR signaling pathway via both short-term and long-term regulation mechanisms.

Insulin is the major hormone controlling critical energy functions such as glucose and lipid metabolism. Insulin elicits a diverse array of biological responses by binding to its specific receptor [4]. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that includes the IGF (Insulin-like Growth Factor) receptor. Insulin has diverse effects on cells including stimulation of glucose transport, gene expression and alterations of cell morphology . The mTOR (Mammalian Target of Rapamycin) is a 289-kDa serine/threonine protein kinase is an intracellular amino acid sensor that responds to insulin via the PI3K/Akt pathway (Wullschleger *et al*., 2006). For mTOR to activate its signaling cascade, it must form the Ternary complex mTORC1 (mTOR Complex-1) and mTORC2 (mTOR Complex-2). Rapamycin-sensitive mTORC1 controls several pathways that collectively determine the mass (size) of the cell. Rapamycin-insensitive mTORC2 controls the actin cytoskeleton and thereby determines the shape of the cell [6].

Autophagy is regulated by the insulin-amino acid-mTOR signaling pathway. Insulin inhibit autophagy in two ways: first by activating mTOR in synergy with amino acids (Codogno and Meijer, 2005), which results in the phosphorylation and inhibition of the protein kinase (ULK1) which plays a key role in autophagosome formation. The second pathway is via Akt/protein kinase B-mediated phosphorylation and inhibition of the forkhead transcription factor FoxO3, which is responsible for the expression of Autophagy related genes (Atg) involved in autophagosome formation [7].

There are more evidence showing that increase in insulin signaling reduces lifespan, in the same way when insulin signaling in reduced lifespan increases . It's not clear that up to what extent this reduction increases the lifespan. Many experiments proved that caloric restriction leads to decreased insulin signaling which resulted in lifespan extension in animals like C.elegans, Dorsophila and rodents. The excess caloric intake and prolonged physical inactivity forced insulin to increase from normal to high plasma concentration in order to maintain normal plasma glucose. The consequent and sustained hyperinsulinemia results in insulin resistance, which therefore paradoxically becomes a common feature not only of elderly patients but also of young people. This is worrisome in light of the evolutionary theory of aging. As discussed early that hyperinsulinemia inhibits autophagy, which is the main reason for abnormal aging.

As we know insulin is the main hormone in the growth and proliferation during early development, it also helps in mitochondrial biogenesis by activating mTOR and PGC 1 α. Later this acts in negative way by inhibiting autophagy, decreases lifespan and cause abnormal aging. So Insulin signaling has both positive and negative regulation. During aging insulin signaling is more predominant, so the repair mechanism Autophagy does not happen properly. As there are more evidence making the above statement acceptable. The reason behind this mechanism is still not clear. When insulin signaling reduces, lifespan increases and in which way this signaling happens is unknown. In this study we tried to determine the effect of hyperinsulin signaling on lifespan and its key regulation in normal condition of aged wistar rats.

II. MATERIAL AND METHODS

A. Animals and induction

Experiments were conducted in accordance with guidelines approved by the institutional animal ethical committee. Young (3 months old) and aged (18 months old) male *albino* rats of Wistar strain (King Institute, Chennai) were used throughout the study. The animals were acclimatized for 1 week and then segregated by marking. Marking was done using 1% alcoholic turmeric solution. The young rats were barrier housed four per cage and aged with two per cage at a temperature of 22 ± 3ºC in light controlled environment with a 12:12 h light-dark cycle, and provided free access to food and water. The animals were divided into two groups- **Group I**: young control rats and **Group II**: aged control rats and each group consisted of five animals. All surviving animals in the study were subjected to terminal sacrifice after retro-orbital blood collection. The animals sacrificed at term were fasted overnight (water allowed), weighed and exsanguinated under ketamine anaesthesia and were subjected to gross necropsy after proper bleeding using intracardial perfusion method with PBS. Brain tissue were excised and frozen immediately in liquid nitrogen followed by storage at -80°C until analysis.

For insulin analysis, about 500 µl of blood samples obtained by retro-orbital bleeding were centrifuged at 4°C immediately for the serum to separate and stored at -20°C until analysis. The insulin levels were determined using ELISA assay kit.

B.Monitoring of Glucose Tolerance

Initial studies on the efficiency of glucose clearance from the blood in young and aged rats were carried out by measuring overnight fasting glucose levels using blood glucose analyzer kit (SD check) to check for the variations in the glucose uptake in young and aged rats. Further tests were carried out using Oral glucose tolerance test. A glucose tolerance test is a medical test in which glucose dose is given and blood samples are taken afterward to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance, and sometimes reactive hypoglycemia and acromegaly, or rarer disorders

of carbohydrate metabolism. OGTT was performed on Wistar rats by giving an oral dose of glucose (2g/kg body weight of rat) and blood glucose levels were monitored using the blood sample taken by tail vein bleeding at 0, 30, 60, 90, 120 and 150 minutes. The response pattern was recorded and graphically represented.

C. Insulin Elisa

It is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidise-conjugated anti-insulin antibodies and anti-insulin antibodies bound to micro plate wells. Wash step removes the unbound antibodies. The bound conjugate is detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

D. Tissue Collection and Processing

Once rat is anaesthetized, the abdomen is wiped with ethanol. The mouse was placed on corked surface with abdomen facing up. The skin was grabbed with forceps at the level of the diaphragm and cut to expose the liver. A lateral cut was made and then upwards cutting through the ribs. The flap was lifted and the cut was made until the heart was easy to access. Then butterfly needle was placed into the left ventricle and PBS was injected at no higher than 0.5 ml/min of flow. Then immediately the right atrium was cut. The buffer was continued until the liver changed into a pale colour and the perfusion was stopped once the liquid flow is free of blood. At this point, required quantity of brain tissue was collected for western blotting. The mouse was dipped in liquid nitrogen and kept in a sealable bag and kept at -80^{o}C. The tissue homogenization was carried out using cell lysis buffer (Cell Signaling). The samples were then diluted with cell lysis buffer to uniform protein concentration, aliquoted and stored at – 20 deg C.

E. Western Blotting

Western blotting is a powerful and commonly used tool to identify and quantify a specific protein in a complex mixture. Protein estimation was carried out by Lowry method. Protein samples were first electrophoretically resolved by 12% SDS-PAGE and then transferred to the PVDF membrane by electroblotting. Following a blocking step, the membrane was probed with monoclonal primary antibody that were raised against the antigen in question. After a subsequent washing step, the membrane was incubated with an HRP-conjugated secondary antibody. Blots were developed by using chemiluminescent film after incubating the membrane with Luminol and H_2O_2.

F. Data Analysis

Data for each variable are expressed as the average ± SD and significance of the differences between mean values were determined by Student's t-test. Probability (p) values of less than 0.05 were considered significant.

III. RESULT AND DISCUSSION

A. Analysis of association of aging with glucose intolerance:

The accumulation of dysfunctional mitochondria is a hallmark of aging process [8]. Due to its significance in glucose metabolism, the mitochondrial dysfunction is reported to alter insulin-glucose metabolic axis and the development of diabetes in aged populations . Since insulin acts as an anabolic hormone, it reduces the cellular catabolic processes. Autophagy is one of the major intracellular catabolic pathways responsible for the removal of damaged organelles and/or protein in response to stress and, thereby, maintains cellular homeostasis. The negative influence of insulin signaling on autophagy allows us to hypothesize that the age associated decline in mitochondrial function is responsible for inhibition of autophagy by altering insulin signaling pathway. To determine whether aging is associated with alterations in insulin signaling, fasting blood glucose levels were measured in young and aged rats using glucose oxidase method. The result shows that there is no significant change in blood glucose levels between young and aged rats (Fig.1A) and this was consistent with the previous report. Since, the glucose levels are regulated by the hormone insulin; it is possible that the changes in the levels of insulin may also influence the observed blood glucose levels in aging. To determine whether aging is associated with the changes in insulin secretion/response both OGTT and insulin levels were measured. The results from OGTT shows that

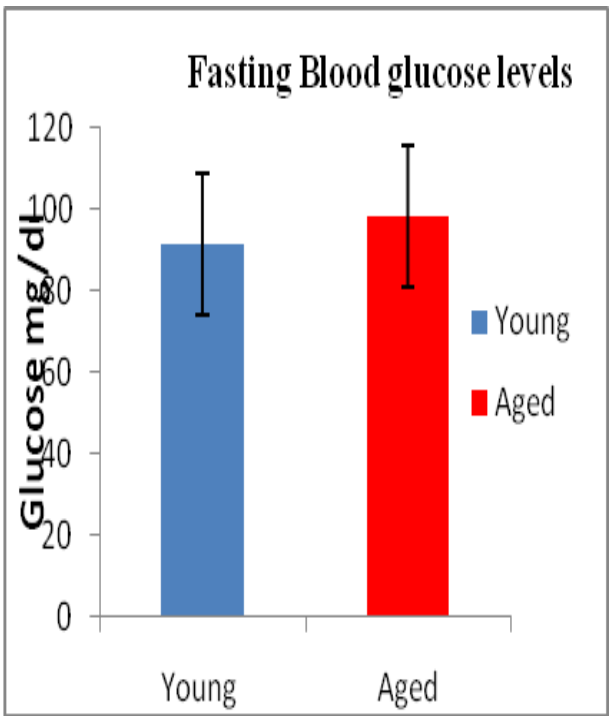


Fig.1A Fasting glucose levels in young and aged rats

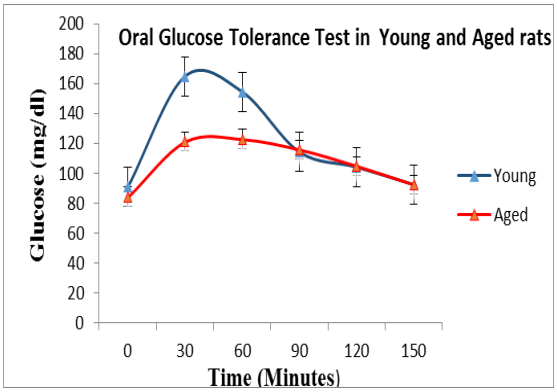


Fig.1B Oral glucose tolerance test in young and aged rats

A. Analysis of fasting Insulin level in ageing

To know whether the change in glucose tolerance of aged rats at 30 and 60 min is due to the change in insulin secretion/response, the serum insulin levels was measured in young and aged rats using indirect-ELISA method. The data indicates that there was no change in the fasting serum insulin levels of aged rats when compared to young rats (Fig. 2). This is also consistent with the earlier report stating that Aged rats present an overall insulin insensitivity in spite of normal fasting blood glucose and insulin concentrations and without developing glucose intolerance as observed in OGTT (Escrivá *et al.* 2007).

The mean glucose values do not differ significantly during 0, 90, 120 and 150 min time points in aged rats when compared with young rats (Fig. 1B). Interestingly, the aged rats (p<0.05) maintained a low blood glucose profile at 30 and 60 min intervals than young rats leading to the possibility of elevated insulin response which acts to clear the glucose levels before reaching the 30 min time point.

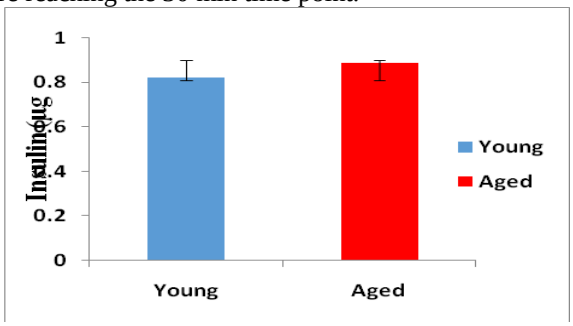


Fig. 2 Insulin levels in young and aged rats

The data are presented as mean±SD (n=5) of young and aged rats. *(p<0.05) Vs. Young rats.

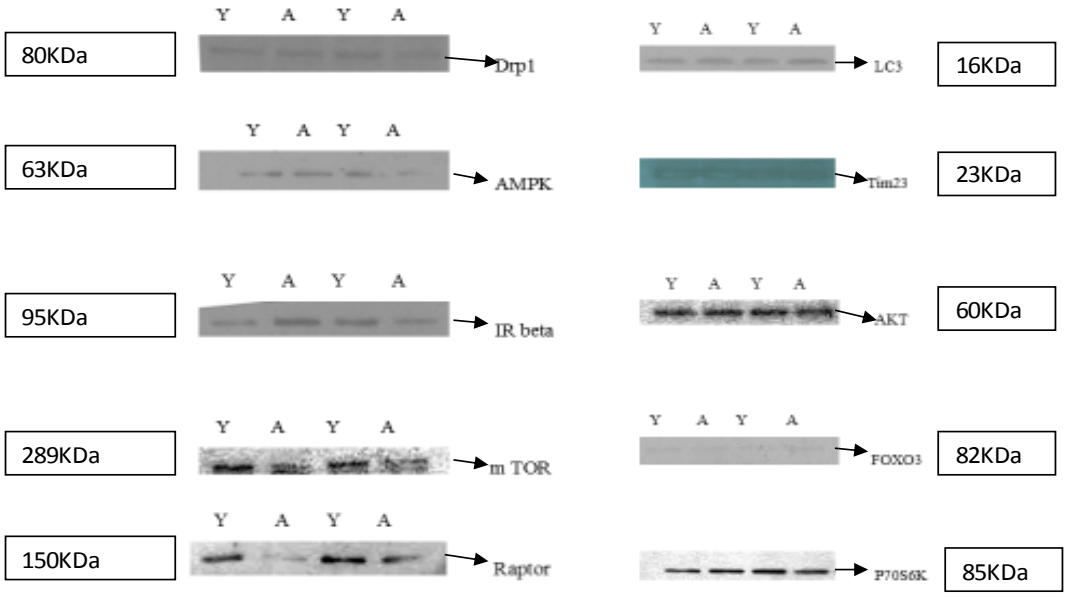
B. Expression analysis of Insulin signaling molecules, autophagy and mitochondrial biogenesis markers in aging

The ligand induced signaling in a cell/tissue is determined by the ligand, receptor and downstream signaling molecules. To determine whether the decrease in insulin concentration in aged rats is associated with the change in insulin signaling in brain tissues, the insulin receptor concentration as well as the expression of downstream signaling molecules was quantified using immunoblot. The results shows that the expression of insulin receptor β and the downstream candidates of insulin receptor such as AKT and p70S6K are not altered during aging (Fig. 3). But interestingly the key mediator of AKT and p70S6K signaling viz., mTOR and mTORC1 complex component Raptor was significantly reduced in aged brain. This finding when correlated to the normoinsulinemic condition prevailing in serum, shows that the downstream insulin signaling in aged tissues are independent of the the serum insulin levels and are differentially altered at the level of AKT and mTORC1 signaling.

Since insulin signaling negatively influences autophagy pathway thorugh inhibition of FOXO3, the levels of FOXO3 and autophagic marker such as LC3-I to LC3-II conversion was determined by immunoblot. The results shows that the levels of autophagic markers are not significantly different between young and aged rats (Fig. 3). Further the levels of mitochondrial biogenesis marker viz., Tim 23 was not altered in aged tissues. Though mTORC1 downregulation is expected to result in an elevated autophagy response in aged tissues, the absence of changes in autophagy markers suggest that AKT mediated suppression of autophagy at FOXO3 level could compensate the possible elevation of autophagy in aged tissues.

C. Analysis of mitochondrial dynamics during aging

Mitochondrial architecture regulated by fusion and fission events are also reported to influence the removal of mitochondria through autophagy. In general, the mitochondria that undergoes fusion resist its entry into autophagosome and survive under starvation conditions to produce energy from cellular waste. Since aging is associated with the accumulation of ‘giant’ dysfunctional mitochondria , it is possible that the dysregulation of mitochondrial fusion-fission may play a role in its accumulation. To know whether aging is accompanied with the change in dynamics, the



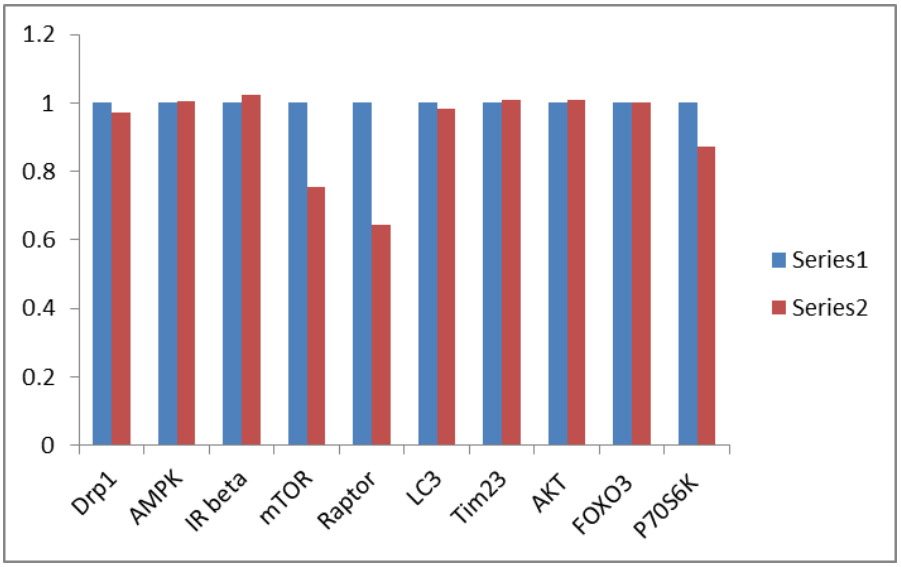


Fig.3 Immunoblot analysis of insulin and autophagy signalling markers.

levels of fission marker (Drp1) was measured by immunoblot. The result shows that there is no significant change in the total levels of both Drp1 in aged rats when compared with young rats (Fig. 3).

IV. CONCLUSION

The regulation of insulin signaling is key to maintain cellular homeostasis and this is achieved at multiple levels such as mRNA expression, protein synthesis and post-translational modifications. This preliminary study primarily targets the levels of protein molecules involved in insulin signaling, autophagy and mitochondrial dynamics. The observed insignificant changes in the autophagy markers in young and aged brain show that these signaling pathways are differentially regulated in the AKT – mTORC1 level irrespective of the serum insulin levels. Owing to the predominance of postranslational modification in the insulin signaling network, this lead could be further evaluated based on the post-translational modifications of these signaling molecules.

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