Chapter I

Introduction

Cells

The “cell” derived from Latin word cella, meaning "small room" is the basic structural, functional, and biological unit of all known living organisms. A cell is the smallest unit of life that can replicate independently, and cells are often called the "building blocks of life". In 1665, Robert Hooke observed tiny, empty spaces confined by walls in thin slice of cork resembling to cells inhabited by Christian monks in a monastery and called it “cells”, the “biological unit of life” (Hooke, 1665). The number of cells in plants and animals varies from species to species and humans contain more than 10 trillion cells. Dimension of most plant and animal cells vary between 1 to 100 µm and are viewed under microscope only.

Cellular homeostasis

Cells are self-sustaining biological unit of life with the capacity to produce required proteins, energy molecules, basic bimolecular building blocks like amino acids, fatty acids to survive and protect from invasion of pathogens, exposure to stresses, and divide. Formation and degradation of biomolecules is continuous and constant as per the need and state of the cells. There are millions of biomolecules involved in intricate balancing cascades of this production and degradation in each and every cells in our body. This phenomenon of intracellular balancing of cellular environment with optimal level of biomolecules is termed as cellular homeostasis. Cellular homeostasis is the most important indicator of balanced cell signalling in a healthy person. It is achieved by balancing turnover and biosynthesis of molecules. Cells often face multiple stresses which disturb this basal harmony and to counteract this scenario cells have an adaptive mechanism of autophagy. Autophagy is a major catabolic process conserved across the species which is predominantly regulated by hormones and amino acids. It is responsible for the delivery of cellular materials comprised of bulk and non-functional lipids and proteins, damaged and leaky organelles, infectious foreign particles etc. to the lysosomes for degradation. Autophagy is thus designed to protect cells from internal and external stresses and the beneficial factor about it is the recycling of reusable building blocks as simple amino acids. Therefore, it supplements cells with energy requirement and thus categorized as pro-survival mechanism. It is required for neonatal development. Activation of one or another autophagic pathway under different cellular conditions, plays an important role in making cells adapting to new environmental changes. Various forms of autophagy have their own attributes depending on their being selective or non-selective in substrate recognition for degradation, but they shares common steps as well. This interdependence of the autophagic pathways confers to the lysosomal
degradation system both specificity and flexibility on substrate selection and degradation.

There are instances when autophagy is defective, insufficient, blocked or excessively active. These conditions have deleterious effects on cellular harmony, resulting in a broad spectrum of diseases, for example, neurodegeneration, fatty liver disease, obesity, pancreatitis etc. When autophagy machinery gets either excessively active or diminished degradation activity, it disturbs homeostasis and often leads to uncontrolled autophagy flux or autophagosomal burden. This further gives rise to various neuro-inflammatory stimuli as in the cases of Parkinson’s disease, Alzheimer’s disease, inflammatory nervous system ailments, sclerosis and associated dementia. Also, aberrant inhibition of autophagy has been linked to age-related disorders including type II diabetes (Barzilai et al., 2012). Certain diseases exploit autophagy machinery for their spread and sustenance as is the case with cancer. Cancer cells have very low nutrient availability and to survive they manipulate autophagy to harness energy supplements (Liang et al., 1999).

There are more than 31 autophagy genes discovered so far and some mammalian counterparts are well known. Beclin1, LC3B are autophagy proteins involved in induction and autophagosome formation respectively. Beclin1 assists Vps34 complex in activating its catalytic activity and initiate autophagy by starting nucleation at PAS (pre-autophagosome structure). mTOR (mammalian target of rapamycin) governs the nutrient sensing and signalling by Akt phosphorylation and negatively regulates autophagy byULK1 phosphorylation under sufficient nutrient availability (Zhao & Goldberg, 2016). LC3B is required for autophagosome-membrane formation and expansion.

p62 is a nuclear sequestosome protein and acts as receptor for cytosolic elements to be delivered at autophagosomes. On the other hand, LAMP2B is a lysosomal membrane protein involved in proper autophagosomes-lysosome fusion. Other than these, there are several proteins which are now found to be involved in autophagy. DRAM1 is a p53 regulated protein which induces autophagy in p53 dependent manner. NF-κB has multiple gene targets and some are involved in autophagy. Generally, basal stimulus for autophagy is very mild but to exert any visible change in signalling, cells require stress stimuli. Typical stresses are starvation, lack of certain nutrient, infection, etc. There are several chemical reagents which are used to induce autophagy, for example, doxorubicin and TNF. TNF is an inflammatory cytokine and activates autophagy. Reports suggest their use as chemotherapeutic agents. However, in most scenario, these agents are found to have cardiotoxicity, high oxidative stress and mitochondrial dysfunction, limiting their use (Dirks-Naylor, 2013; Lalazar et al.,
2016). It is also evident from studies that these agents may deregulate autophagy. Certain molecular changes in signalling also result in formation of metabolic byproducts which are harmful to cells and induce secondary signalling cascades, disturbing entire cellular homeostasis. Glycolysis intermediates, fatty acid synthesis participants and other cascade members give rise to a number of active metabolic modifiers, like methylglyoxal and glycoaldehyde. These modifiers irreversibly convert any protein or lipid to non-functional entity which is termed as Advanced glycation endproducts (AGE).

AGE is one of the major cellular stress originated by natural reactions of metabolic byproducts. It forms non-enzymatically in our body in response to high glucose availability and reduction-modification by methylglyoxal or glycoaldehyde etc. Mostly this process affects long-lived proteins and lipids but irreversibly which renders them non-functional or dysfunctional. Therefore, it is damaging to the proper functioning of cellular system. Collagen modification affects flexibility of muscles; RBC elasticity gets disordered; retinal protein modification can cause loss of sight; extracellular matrix protein modification causes membrane stiffness and disturbed membrane transport; AGE mediated arterial damage, irreversible vascular damages amounting to heart failure and inflammatory arthritis are few AGE promoted deleterious effects. There are several health complications associated with AGE mediated signaling including neurodegeneration, liver, diabetes, obesity, and kidney, and few more.

Prevalence of diabetes has increased over the decades as a pandemic and is often associated with complications of obesity. This dual public health problem is preferably termed as “diabesity”. The World Health Organization (WHO) data predicted that there are over 700 million obese people worldwide and 70% of diabetic patients belong to developing country (Yaturu, 2011). Type II diabetes is the most common form constituting 80% of total diabetes patients. Mortality and morbidity in diabetes is frequently linked to irreversible vascular damages mediated by AGE. (Hobbs, 2006). Current drugs available for diabetes treatment like glipizide and glimepiride (Sulfonylureas), repaglinide and nateglinide, or thiazolidinediones class of drugs have been reported with obesity issues. Also, autophagy deficit and AGE mediated signalling is reported in various neurodegeneration disorders, Prions diseases, however, any mechanistic connection between the two has yet to be revealed (Li et al., 2012). Therefore, it becomes imperative to find any link if exists in autophagy and AGE. This becomes the base to start our study. AGE mediated signalling is involved in lipid metabolism and therefore, hyperglycemia, a common feature of diabetes puts these patients
at double risk of associated health complications of obesity and organ failure. AGE accumulation creates a cytotoxic load on cells which exerts multiple signalling responses through its receptor RAGE. AGE-RAGE signalling increases ROS and inflammation, speeding the ageing process, damaging organ functions and eventual loss by augmenting DNA damage, diminishing repairs, and manipulating regular check-point controls which results in aberrant cell division or loss of function to many processes. NF-κB and other transcription regulators like p53, SREBP, AP-1 are activated in response to AGE binding and ROS generation.

NF-κB is considered prototypical inflammatory signalling regulator because of its involvement in regulation of pro- and anti-inflammatory chemokines, cytokines and various adhesion molecules (Lawrence, 2009). It participates in regulation of multiple cellular processes including cancer metastasis, differentiation, cell death and survival. Vast range of stimuli coalesce on NF-κB activation. Therefore, cells have multiple positive and negative regulatory mechanisms for NF-κB dependent signalling and gene transcriptions. Deviation from these tightly regulated controls under stress results in loss of signal coordination and resultant uncontrolled transductions, causing irreversible damages to cells. NF-κB also regulates autophagy machinery upon exposure to different stimuli (Lin et al., 2015) through regulation of several autophagy genes. Beclin1 and p62 are two of them. It shows synergy with various transcription factors and modulates many parallel signalling pathways. SREBP and p53 are two common collaborators involved in myriad signalling cascades. Inversely, hyperactive NF-κB interferes with regulatory functions of other transcription factors like p53 through MDM2 transcriptional regulation (Oeckinghaus et al., 2011), masking effective controls over cell cycle deregulation, DNA damage and apoptosis. This results in rapid ageing, multiple organ failure, memory loss and neuronal cell death. Several reports suggest that both the transcription factors act antagonistically and p53 also can repress NF-κB activation however, upstream signalling remains unaffected (Ryan et al., 2000; Shao et al., 2000; Murphy et al., 2011).

p53 is a tumor suppressor involved in apoptosis, cell cycle regulation, DNA damage and repair signaling cascades, and carbohydrate and amino acids metabolism. Recent studies have given new insights of p53 regulatory effects on autophagy. p53 induces autophagy through its target gene DRAM1 (damage-regulated autophagy modulator) which was originally described as apoptotic effector (Crighton et al., 2006). Reports also indicate that p53 regulate autophagy through LC3B protein expression and exacerbate DNA damage
induced autophagy (Scherz-Shouval et al., 2010). p53 also determines the fate of cells by interacting with downstream regulators as is the case with Beclin1-p53 interaction which is involved in determining embryonic cell fate by reducing autophagy level (Tripathi et al., 2014). However, evidences suggest that cytoplasmic p53 inhibits autophagy (Hoshino et al., 2013; Tang et al., 2015). Reports also indicate its involvement in lipid catabolism. Glucose deprivation activated p53 induces guanidinoacetate N-methyltransferase (GAMT) expression which is involved in creatine synthesis. This way p53 assists in supplying energy through fatty acid oxidation as an alternative for glycolysis in starvation (Goldstein & Rotter, 2012).

The other transcription factor which is primarily involved in lipid metabolism and glycolysis is SREBP (sterol regulatory binding protein). It is responsible for activation of fatty acyl synthase (FAS), acetyl CoA carboxylase (ACC) and stearoyl CoA desaturase (SCD) enzymes which participates in synthesis pathway. Lipid synthesis upon ample availability of carbohydrates or proteins as well as its degradation upon starvation is finely balanced by coordinated signalling of SREBP and its target genes. MTOR recognizes satiety via active AKT, PKC and insulin signals but regulatory deficit results in aberrant gluconeogenesis and lipogenesis in cells (Hagiwara et al., 2012).

Lipogenesis is the process of synthesis of lipids from metabolites of glycolysis pathways mainly sugars with the catalytic activity of FAS, ACC and SCD. Lipogenesis mostly stays in harmony with lipolysis of lipid droplets, where free fatty acids are released through enzymatic breakdown when energy crisis arises in the cells (Mahali et al., 2014). However, disturbance in signaling based on changing stimuli often results in high accumulation of lipids causing obesity. Autophagy is meant to maintain homeostasis but when mTOR signalling gets deregulated it may result in excessive autophagy or complete blocking which leads to unchecked glucose and lipid production or accumulation. High lipogenesis and defective autophagy are exhibited by cells with deregulated coordination of upstream signalling heads including SREBP and NF-κB. All these alterations sum up to exacerbate diabetes associated obesity complications. Therefore, SREBP-mediated signalling requires tight control with the help of other factors. NF-κB assists SREBP functioning (Li et al., 2015). Accumulation of lipids often hamper neuronal functions in autophagy deficit neurons. This is also observed as side effects of diabetes drugs. Various pharmacological blockers are used to check this accumulation including thiazolidinediones. However, drug prompted obesity cases are often cited by the patients and are supplemented with sterol pathway
blockers like novastatin. This chemical blocks HMG-CoA and fatty acyl synthase activation thereby inhibiting lipogenesis. Growing number of diabetes and obesity associated complications, diminished mental ability or memory loss in ageing people with inflammatory symptoms require further studies to understand elaborated signalling cascades coalescing to lipid accumulation mediated deleterious effects, mechanism of their collaborations to other regulators are critical to design effective therapeutics.

**Rationale of the study**

Now a days, with increasing trend of sedentary lifestyle, we are getting more prone to AGE associated health complications and resultant ailments. This is a pressing situation to explore futuristic approach to counteract such health hazards and this can be underlain by mechanistic studies which will give better insights into how AGE works to initiate heath troubles. AGE promotes ROS and inflammation. Unchecked inflammatory stimuli activates deregulated signalling cascades involved in damage repair, cell division or neuronal growth. All these alterations are also linked to autophagy deficiency or hyperactivation. Involvement of AGE in cell survival and cell death, activation of autophagy in both scenario but no reported studies to link the two processes prompted us to explore and investigate further that how AGE affects autophagy and associated cellular signallings.

Several recent studies are focused on finding natural substitutes/ biomolecules or small molecules which induce autophagy in cells and conditions with low autophagy flux. However, very less mechanism based studies have been done to answer how autophagy is activated by cellular metabolic byproducts and its effects. In some cases, drugs available for treatments show side effects of obesity in the patients. Therefore, to find new non-harmful, natural biomolecules or compounds which may help subsidize the severity of these disease complications without any side effects, we need to understand the underlying mechanism. This study will provide avid insight into the regulatory mechanism of AGE induced autophagy and its impairment resulting in apoptosis.

**Hypothesis**

- We hypothesize the involvement of NF-κB as master regulator of AGE induced autophagy and lipogenesis.

- NF-κB upregulation is associated with AGE-mediated autophagy impairment in p53 negative cells.
Autophagy and lipogenesis are mechanistically linked

AGE activates apoptosis upon autophagy impairment.

**Objectives**

To check the validity of our hypothesis, we have addressed the following objectives in this study-

- AGE mediated autophagy and its regulatory mechanism.
- AGE mediated lipogenesis, its regulatory mechanism and link to autophagy.
- AGE mediated autophagy impairment, its mechanism and switching mechanism to apoptosis.

**Inferences**

Briefly, this study presents the following conclusions:

- AGE induces autophagy which is regulated by an intricate signalling orchestration of NF-κB with Raf kinase and PKC conveying regulatory messages through MAPK pathway.

- AGE mediated lipogenesis is similarly regulated by NF-κB and Raf kinase however, we found no mechanistic interlink between the two processes- autophagy and lipogenesis.

- Also, AGE impairs autophagy in p53 negative cells which results in switching to apoptosis through Beclin1 cleavage.