“Life” sounds so simple but is the display of complexity in our universe. From unicellular to multicellular, every organism has its own set of complex molecular set-up, some understood well by rigorous research and studies of several pioneer and some still under the wrap waiting to be uncovered. Every living being is designed to sustain and endure all sorts of stresses but, with loads of molecular burden come a point, which determines our capacity to survive or succumb to the end. Every individual cells respond differently to a single stress because of differential gene expression. It is the basal signalling cascades which balance the cellular chaos to homeostasis.

Despite being equipped with complex machinery to fight deleterious effects of stresses which bring molecular changes in cells, we age and some of us age faster than the others, our organs’ functions deteriorate and eventually life is lost. The question arises how we age or why certain groups of people are more prone to faster ageing. Cell signalling is accountable for every singular change at molecular level. Some external or internal factors predispose us to a set of changes which is not evident in others. Deregulated signalling often alter physiological response towards specific stresses which in absence of homeostasis or slow housekeeping maintenance, irreparably damage cells leading to loss of functions. Once a particular set of genes lose its function, it affects the functional attributes of multiple others and gradually cells lose viability and this happens in hierarchy leading to complete functional loss of organs.

Now a days, with increasing fondness 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 against such health hazards and this can be underlain by mechanistic studies which will give better insights into how AGE works to initiate heath troubles. Accumulated advanced glycation end products have been shown to aggravate several ailments in ageing people and diabetic patients. An understanding of the molecular mechanism of these detrimental effects is required for future effective therapy. Obesity is one of the key phenomenon under these conditions because of decreased metabolic activities. Presently, several peroxisome proliferator-activated receptor analogs, like glitazones, are in use to treat diabetes which often have obesity as a side effect. Autophagy is a housekeeping mechanism cells are devised with. The main business of autophagy is to get rid the cells of any cellular debris including mis-folded proteins, excess of lipids, injured organelles which may cause cytotoxicity harming neighbouring cells and disturbing multiple signalling pathways.
There are lots of manipulative cellular factors which breach the central purpose of autophagy to serve in beneficial light to the cells and start secondary signalling cascades upsetting the homeostasis and creating cytotoxicity pool. Once defective, autophagy becomes a physiological burden and then this event signals second line of defensive clearance body to take charge- “apoptosis” comes into the picture. We found AGE induces autophagy and lipogenesis, so uncovering the detailed mechanisms correlating AGE, autophagy and lipogenesis might help to find a way to regulate AGE-mediated deleterious effects like obesity. The involvement of peroxisome proliferator-activated receptors in lipid accumulation and obesity or its correlation with autophagy has yet to be explored.

Methylglyoxal- glycated HSA was used in this study as AGE and the concentration used was within the limit of the physiological concentration in the serum. HSA itself does not exerts any cell signalling, but glycated HSA employs multiple downstream signalling upon RAGE binding. RAGE is an immunoglobulin (Ig) superfamily member, a transmembrane protein containing extracellular domain (three Ig-like domains) with highly charged cytoplasmic tail. Downstream signalling perpetuates inflammatory signals either leading to tissue repair or chronic inflammatory damages and thus is accounted for pathological conditions in various diseases like atherosclerosis, Alzheimer’s disease, diabetic nephropathy, respiratory and liver disorders. With the aim of finding mechanistic links, we checked our various hypothesis. We observed potent induction of autophagy by AGE, greater than the known inducers like TNF or doxorubicin, as determined by examining fluorescence-labelled autophagosomes, its quantification and the increase in autophagy markers like Beclin1, DRAM1, and LC3B by Western blot analysis. AGE induced autophagy is not restricted to specific cell types, but the degree of induction depends on the amount of its receptor, RAGE. AGE-mediated autophagy is significantly inhibited upon blocking PI3K by wortmannin. AGE-RAGE interaction activates type III PI3K, which further relays to downstream signalling molecules to exert AGE-mediated functions. If RAGE recruits PI3K, a membrane-anchored enzyme, upon ligand interaction, or its mediator molecules require further investigation. Bafilomycin A1, a known maturation blocker, potentially retained the AGE-induced autophagosome, further signifying AGE mediated autophagy induction. AGE induces NF-κB through generation of reactive oxygen species, followed by IKK activation. Treatment with BAY 11-7082, an IKK complex inhibitor and potent NF-κB activation blocker or transfection with dominant negative IkBa abolished autophagy. NF-κB regulates several cellular processes through gene transcriptions. Beclin1,
Chapter VII

Conclusions and Summary

DRAM1, and LCBs are involved in autophagy. The expression of Beclin1 is dependent on NF-κB, and inhibition of NF-κB subsequently represses autophagy. AGE mediated NF-κB activation regulates autophagy. Sorafenib, a Raf kinase inhibitor, suppressed downstream kinases like ERK and p38 MAPK, and reduced autophagy. Even the PKC inhibitor, which suppressed ERK and p38 MAPK, partially blocked autophagy. NF-κB inhibitor together with Raf kinase inhibitor resulted in complete blockage of AGE-mediated autophagy. Kinase assay revealed reduction in MEK1 phosphorylation in cells lysate with blocked PKC indicating PKC control over Raf kinase activity. Therefore, upstream signalling molecules, either PKC and/or Raf kinase, contribute to AGE-mediated autophagy induction via activation of the downstream molecules p38 MAPK and ERK. AGE promotes lipogenesis, as shown by lipid droplets accumulation, and this is correlated with the autophagic event. Although we are getting these events concurrently upon AGE-stimulation, exactly how autophagy enhances lipid accumulation demands further investigation.

Bafilomycin A1, an autophagosome maturation blocker, sustained the level of autophagosomes and retained the amount of lipid particles in the cells. Autophagy is designed to clear unwanted cellular content, such as non-functional organelles, mis-folded proteins, and lipids, under tight surveillance of signalling molecules. Several reports have proposed that autophagy machinery degrades lipid droplets, although we did observe retention of lipid droplets in AGE-stimulated autophagy. Caffeine and epigallocatechin gallate clear lipid droplets by activating autophagy assisted lipolysis. We observed that glucose-mediated lipogenesis dictates cells for autophagy initiation, whereas AGE augmented autophagy prior to lipid accumulation. This observation exemplified that AGE-mediated autophagy is assisting both the processes—lipogenesis on one hand and lipolysis on the other however, the outcome is accumulation. This can be addressed as AGE might promote lipogenesis and autophagy independently, but the amount of lipid synthesized is more than the consumption in the cells via autophagy. AGE also induces cell death. Accumulated lipid particles might elevate AGE-mediated cell death which would be a good extension to study. Lipogenesis and lipolysis are interconnected processes which depend on the expression of SREBP. Processing of SREBP via the Golgi-endoplasmic reticulum network is required for its transcriptional activation which then activates lipogenesis by activating gene transcriptions of FAS, ACC or fatty acyl CoA. We did not observe any interference in SREBP processing upon AGE stimulation but SREBP expression increased via NF-κB activation. Statins (e.g. novastatin) are a group of compounds which
competitively obstruct the activation of enzymes involved in lipogenesis. Novastatin is known to inhibit SREBP transcriptional ability, thereby interrupting lipogenesis, but has not hampered AGE-mediated autophagy significantly in our study.

The inhibitors of NF-κB, PKC, or Raf kinase partially supressed lipid accumulation. Earlier we showed that PKC inhibitor blocked Raf kinase and hence, partial inhibition of AGE-mediated autophagy and lipogenesis by the PKC inhibitor which further supports the involvement of Raf kinase. The inhibitors of Raf kinase and IKKs in combination entirely suppressed lipid accumulation, signifying that these two pathways are involved in AGE-mediated lipogenesis. Downstream inhibitors were also able to reduce AGE-mediated lipogenesis to some extent. However, upstream inhibitors such as BAY and sorafenib were able to block lipogenesis effectively and were comparable with the novastatin effect. We also presented data demonstrating that the AGE-RAGE interaction activates signalling cascades: PKC, Raf kinase, MEK1/2, ERK, p38, MAPK, and IKKs. These events subsequently enhance NF-κB and SREBP transcriptional activity, which increase lipogenesis and autophagy. IκBα-DN-transfection reduced SREBP expression upon AGE stimulation and similarly decreased AGE-mediated lipid accumulation. This supports our hypothesis that NF-κB definitely is the principal regulator in AGE-mediated lipogenesis.

Restraining autophagy by ATG7 and ATG12 shRNA transfection resulted in minor decrease in AGE-induced lipid accumulation, indicating fractional clearance of lipids by autophagy. Concurrence of lipogenesis, lipolysis and autophagy perhaps upset the balance of the overall cytoplasmic lipid droplets. The accumulated lipids signal the autophagy machinery to clear them up, but the AGE-mediated induction of lipogenesis dominates autophagy mediated lipolysis leading to its accumulation. Therefore, AGE-mediated detrimental effects are often observed in ageing people or diabetic patients because of the high accumulation of AGE. There is grave requirement to develop appropriate therapeutics to regulate these signalling pathways, to nullify adverse effects of dominating lipogenesis or to stimulate the autophagic lipolysis mechanism to control the unwanted stockpile hazards of lipid accumulation and obesity.

There are several reports which suggest p53 mollify NF-κB mediated alterations especially in cases of tumourgenesis, glucose metabolism and ATP generation. The p53 is a transcription factor with tumour suppressor activity. It is associated with death receptor and can regulate Apaf1 mediated apoptosis activation, senescence, autophagy and proliferation. Recent reports suggest that p53 interacts with Beclin1 and manipulates its association with
other partner proteins. p53 is also shown to regulate microtubular light chain protein (LC3B). NF-κB transcriptionally regulates Beclin1 and p62. Autophagy becomes faulty in certain settings upsetting the homeostasis. It is known that autophagy starts with activation of Vps34 complex (class III PI3 kinase complex) which requires assistance of Beclin1 to gain its catalytic activity.

Beclin1 can also interact with Bcl-2 family proteins, but interaction with Bcl-2 always hinders the Beclin1 association with Vps34 stalling its kinase activity and thus blocking initiation of autophagy. The p62, on the other hand is a nuclear multi-domain scaffold protein which binds to LC3B and cytoplasmic contents which has to be delivered to autophagosomes for degradation and in turn, gets degraded itself. Therefore, its accumulation in autophagosomes serve as marker for clearance impairment of autophagy. Accumulation of LC3B II is also a well-accepted representation of impaired autophagosomes’ degradation. The other important participant in autophagy is lysosomal membrane protein LAMP2B, which is involved in lysosome-autophagosome fusion and gets degraded with autophagosomes. Therefore, its accumulation flags the impaired degradation. It is also evident from various studies, that high cytoplasmic p53 blocks autophagy and the nuclear p53 expression dictates NF-κB expression, mostly inversely. Faulty or dysfunctional autophagy is implicated in variety of pathophysiological complications including pancreatitis, diabetic complications, obesity, and lysosomal storage diseases, neurite cell death and inflammation, often favouring cell death mechanisms. The amount of AGE increases with ageing, and cases of Prion’s diseases are growing in ageing groups. The deregulated clearance of these defective proteins might have impact on the cell viability, especially the long lived neurons affecting memories and learning aps.

After demonstrating the involvement of NF-κB in AGE mediated autophagy regulation with the coordinated assistance of Raf kinase and MAPK, the curiosity perplexed us to explore its involvement if any, in eliciting cell death signals as NF-κB is known to induce a variety of acute and chronic inflammatory signals causing cell death.

The second section illustrates evidences which advocate that with increase in the amount of AGE, increases the NF-κB expression which gets enhanced by diminished or non-functional p53. We found Beclin1 cleavage in AGE stimulated p53 negative cells which appeared to be an autophagy impairment manifestation. We also observed that NF-κB-dependent gene NEDD4 increases this Beclin1 cleavage which accompanied autophagosomes’ accumulation and pronounced cell death. Based on our preliminary observations, we hypothesized that
impairment of autophagy might be signalling the other clearance mechanism to combat the cytotoxic load created by its deficits. Apoptosis is one such prime death mechanism which rescues the neighbouring cells from the toxic hazards of this burden.

To validate our hypothesis and its correlation with AGE-mediated autophagy, we assessed autophagy index in randomly picked p53 wild type or negative cell lines and found considerably high autophagy index in the p53 negative cells, suggesting accumulation of more autophagosomes in these cells. Beclin1, LC3B and p62, the autophagy markers were in standard range in p53 positive cells except Beclin1 in p53 negative cells. We witnessed cleavage pattern in Belcin1 in the AGE stimulated Saos-2 cells. This atypical result prompted us to investigate and compare the autophagosomes’ accumulation in p53 positive and p53 negative cells. To examine autophagosomes’ clearance, we performed p62, LC3B and LAMP2B immunofluorescence in rapamycin or AGE stimulated p53 negative cells and compared the results to confirm the difference. There was unexpectedly high accumulation of p62 and LAMP2B in the AGE stimulated cells indicating hampered degradation. However, rapamycin exerted no significant changes. This confirmed that AGE treatment alone is accountable for autophagosomes’ accumulation and is restricted to p53 negative cells. We then compared autophagy and its markers in both cell types. p53 negative cells showed evidently high autophagosomes’ staining pertaining to high accumulation rate. This supported the fact that the elevation in autophagosomes’ accumulation in p53 negative cells is resulted from impaired clearance. LC3B puncta, (as detected by immunofluorescence and Western blot) were also very pronounced in AGE treatment than rapamycin in Saos-2 cells, further signifying the retention of autophagosomes in these cells. We wanted to further explore the associated manifestations of this impairment and we observed substantial cleavage of Beclin1 in p53 negative cells and p62 accumulation specifically in AGE stimulation, as rapamycin treatment in both cell types did not yield such cleavage pattern or p62 accumulation. The Beclin1 cleavage was not restricted to HCT116 (p53\(^{-}\)) cells but Hep3B cells also showed similar cleavage of Beclin1 and p62 accumulation confirming our hypothesis that this cleavage is associated with absence of p53.

AGE activates NF-κB through ROI increase and IKKs activation. To determine the mechanism behind this AGE-mediated autophagy impairment specifically in p53 negative cells, DNA binding activity of NF-κB was examined in HCT116 (Wild) and HCT116 (p53\(^{-}\)) cells which revealed significantly high NF-κB activation in p53 negative cells. Likewise, \(p65\) overexpression in HCT116 (p53\(^{-}\)) and \(p53\) shRNA transfection in HCT116 (Wild) cells
caused Beclin1 cleavage upon AGE stimulation. *IkBα-DN* overexpression repressed and *p65* overexpression elevated this AGE mediated cleavage in HCT116 (*p53−/−*) cells. *IKK-WT* transfection too had similar effect on Beclin1 in these cells signifying role of NF-κB in this cleavage. Also, the *p53 shRNA* transfection in HCT116 (Wild) cells showed significant increase in NF-κB expression in these cells indicating tight regulation of NF-κB by *p53* justifying the elevated NF-κB expression in *p53* negative cells. Next, we elucidated the connection between autophagy impairment and apoptosis initiation and for this, we treated cells with pan caspase inhibitor, proteasome inhibitor MG-132 and wortmannin in presence of AGE. We detected Beclin1 cleavage substantially repressed by pan caspase inhibitor and MG-132, indicating the involvement of ubiquitin ligase in cleavage of Beclin1 and its interaction with apoptotic proteins.

The mRNA and protein expression level of NEDD4, an NF-κB targeted E3 ubiquitin ligase was complementary and elevated in *p53* negative cells demonstrating its involvement in ubiquitinating Beclin1 for degradation. Co-immunoprecipitation assays confirmed Beclin1 interaction with NEDD4, its ubiquitination and *p65* interaction. Surprisingly, we also detected Beclin1 interaction with *p53*. Collectively, these observations suggest that changes in Beclin1 interactions determine autophagy induction and its impairment. Perhaps *p53* interaction engages Beclin1, shielding it from NEDD4 mediated cleavage. Numerous reports suggest *p53*-Beclin1 interaction through BH3 domain which manipulates its interaction with Bcl-2 family proteins and affects autophagy. Moreover, Beclin1 interaction to Vps34 is indispensable to activate Vps34 complex which then initiates autophagy. In our study, we detected interaction between Vps34 with Beclin1 in 24 h AGE stimulated HCT116 (Wild) and HCT116 (*p53−/−*) cells but, this interaction was considerably reduced in 48 h or 72 h lysate. This predicts the loss of interaction between both the proteins which releases Beclin1 to interact with other proteins. Conceivably, we found the interaction of Beclin1 with Bcl-2, an anti-apoptotic protein. Engaged Bcl-2 relieves the apoptosis barricade which activates apoptotic cell death routes. This engagement also hinders in Beclin1-Vps34 mediated autophagy activation and thus autophagy gets stalled.

Although, autophagy is a basal pro-survival mechanism cells are gifted with but, the revelation of AGE mediated autophagy impairment in this study provides new ground for further research to tackle AGE associated detrimental signalling and related disorders in ageing, neuroinflammatory disorders, and survival of cancer cells. Thus, this study provides a profound insight into the mechanism of switching between the two indispensable processes.
autophagy and apoptosis, the mechanisms cells are equipped with- “one to conquer and other to defeat” and signify the importance of p53 in altering the signalling cross-talk that determines the choices of processes to follow and which to abandon.

This study therefore broadcasts the importance of AGE mediated signalling in health and diseases.

The highlight of this study are-

- AGE induces autophagy and NF-κB regulates autophagy machinery with the assistance of Raf kinase, PKC and MAPK framework.
- AGE mediated lipogenesis is NF-κB dependent and Raf kinase plays important role in its regulation.
- AGE induces autophagy impairment in p53 negative cells through NF-κB interferences via Beclin1 cleavage by NEDD4 and p53 shields cells from NF-κB mediated impairment.
- Autophagy impairment augments apoptosis in p53 negative cells higher than p53 positive cells.

**Future prospects of the study**

1. Involvement of AGE mediated autophagy impairment in ageing, neurodegeneration and lipolytic or lipogenic disturbances.
2. Identification of interacting partners of Beclin1 which may manipulates downstream signalling cascades.
3. Signalling cascade of ULK1 in autophagy and its interacting partners which may lead to impairment and cell death.