5.1 Introduction

AGE is often shown to accumulate in hyperglycemia where glucose metabolism is disturbed. Excess glucose is often converted into fatty acids by insulin and stored as fats in cells. Due to availability of high level of sugars, the chances of glycation of lipids increase as well. This often gives rise to hyperlipidemic condition with accumulation of these modified lipids. This rise in lipid accumulation results in obesity, a well-known manifestation of diabetes. All these conditions further worsen the health with deteriorating cardiac functions because of lipids blocking arteries, a condition called atherosclerosis. If this condition goes unchecked, often leads to heart failure. Besides this, AGE formation leads to severe tissue damage due to high oxidative stress. AGE binds its receptor RAGE and this starts a chain reaction of several signalling pathways which often are associated with inflammation, major cause of diabetes and age-associated disorders. AGE activates several transcription factors. NF-κB and SREBP are few of these regulatory transcription factors. Recent reports suggest AGE increases lipid droplets through SREBP activation. SREBP, a helix-loop-helix containing transcription factor; is a 120 kDa protein which gets cleaved by active site-1 and active site-2 proteases to release active amino-terminal domain. This is followed by phosphorylation at Ser432 and Ser355 by ERK to activate the SREBP (as a transcription factor). Therefore, ERK is important for SREBP activation on one hand and on the other, ERK itself gets regulated by upstream kinases like MEKK1, MEK1 etc. There are PPARγ analogs which are used to combat the lipid accumulation in diabetes patients however, their side effects restrict their uses and therefore, it is imperative to understand how this AGE-mediated lipogenesis is regulated by SREBP level.

Autophagy is the basal mechanism to clear out any surplus amount of proteins and fats from the cells. We have shown that AGE induces autophagy under NF-κB surveillance through Raf kinase and MAPK pathway. It is obvious to ask question about the involvement of autophagy in lipogenesis or lipolysis and the mechanism of its regulation. This will help in developing and sorting of drug candidates/biomolecules which, with least side effects can be used to control lipidemia in diabetes and old age people.
5.2 Results

5.2.1 Autophagy promotes lipogenesis

Autophagy is responsible for supplying energy by degrading cellular debris and AGE induces autophagy as well as lipogenesis. Therefore, to check the involvement of autophagy in lipogenesis, Oil Red O staining (Mahali et al., 2014) was done in AGE treated HepG2 cells and further correlated with MDC stained autophagosomes. Both lipogenesis and autophagy were remarkably elevated upon AGE stimulation in time dependent manner (Fig.5.2.1A). To validate the possible contribution of autophagy in lipogenesis, Oil Red O staining was again done in presence of autophagy inhibitors and mangiferin, a known inhibitor of AGE-mediated lipogenesis. This revealed dramatic drop in lipid droplets as indicated in images (Fig.5.2.1B) or the mean fold of induction determined from absorbance data of three independent experiments (Fig.5.2.1C).

Figure 5.2.1: AGE mediated autophagy and lipogenesis. HepG2 cells were treated with AGE (100 µg/ml) in two sets for varying time spans as indicated and subjected to oil red O and MDC staining separately (A). HepG2 cells were pre-treated with autophagy inhibitors- BafA1 for 5 h and WM and mangiferin for 3 h followed by AGE stimulation for 12 h and stained with oil red O (B). Absorbance of oil red O at 500 nm were recorded and represented as fold increase extrapolated from three independent experiments in autophagy inhibitors pre-treated and AGE stimulated HepG2 cells (C). Error bars represent S.E.M., Student’s t-test *** p<0.001.
5.2.2 Raf kinase and IKK significantly control AGE-mediated lipogenesis

To identify upstream signalling participants, we shortlisted a few candidates from common survival pathways and assessed their involvement by blocking these one by one. PKC I (PKC inhibitor) or SB (SB203580- p38 inhibitor) and PD (PD098059- MEK1 inhibitor) treatment resulted in partial inhibition of lipid accumulation upon AGE stimulation in HepG2 cells as examined by oil red O staining. BAY (BAY117082) or SR however, were more effective in reducing lipid accumulation in AGE stimulated cells (Fig.5.2.2A). The oil red O staining and absorbance of oil red O stained lipid droplets in BAY and SR (combination) treated HepG2 cells showed most significant reduction in lipid droplets and were comparable with the commercially available inhibitor novastatin, an HMG CoA pathway inhibitor (Fig.5.2.2B and Fig.5.2.2C). These data advocate about the involvement of NF-κB and Raf kinase pathways in AGE-mediated lipid accumulation.

![Image of Figure 5.2.2 A & B: Effect of NF-κB and other kinase inhibitors on lipogenesis. HepG2 cells pre-treated with BAY (2 µM for 3 h), SR (10 µM for 3 h), SB (2.5 µM) and PD (2.5 µM) for 5 h or PKC I (2 µM for 3 h) were stimulated with AGE (100 µg/ml) for 12 h and subjected to oil red O staining. Images were captured and represented (A). HepG2 cells treated with BAY (2 µM) and SR (10 µM) together for 3 h or novastatin (2 µg/ml for 3 h) followed by AGE treatment for 12 h and then oil red O staining was done. Images of oil red O stained cells are represented (B).]
5.2.3 NF-κB and Raf kinase regulated AGE-mediated lipogenesis

Initial results of decrease in lipogenesis by autophagy inhibitors led us to speculate that autophagy is involved in lipogenesis. To inspect this point, autophagy markers were examined by Western blot. AGE-mediated increase in the amount of autophagy proteins-Beclin1 and DRAM1 and fatty acyl synthase (FAS) were markedly decreased in BAY treated cells as determined by RT-PCR (Fig.5.2.3A). Also, as with the BAY and SR pre-treatment, p62 accumulation was observed along with reduction in the levels of Beclin1 and LC3B indicating decrease in autophagy level (Fig.5.2.3B). These data strongly suggest that NF-κB plays vital regulatory function in lipogenesis. However, novastatin treatment did not result in significant reduction of LC3B or Beclin1 (Fig.5.2.3C) but marked drop in lipid accumulation was evident in novastatin treated HepG2 cells as observed by oil red O absorbance (Fig.5.2.3D). Combinatorial treatment with BAY and SR resulted in complete inhibition of AGE mediated lipid accumulation and autophagy index as indicated by absorbance of oil red O staining and MDC staining in HepG2 cells (Fig.5.2.3D). To further support this outcome, HepG2 cells were transfected with IκBα-DN followed by AGE stimulation for 12 h and subjected to oil red O staining. Representative images of lipid particles (Fig.5.2.3E1) and absorbance plot of oil red O

Figure 5.2.2 C: Effect of NF-κB and other kinase inhibitors on lipogenesis. Absorbance of AGE stimulated HepG2 cells pre-treated with BAY (2 µM for 3 h), SR (10 µM for 3 h), SB (2.5 µM) and PD (2.5 µM) for 5 h and PKC I (2 µM for 3 h) was determined after oil red O staining (C).
stained lipid droplets in AGE treated \( \text{IkBα-DN} \) transfected HepG2 cells (Fig.5.2.3.E2) indicated significant decrease in AGE mediated lipid accumulation.

**Figure 5.2.3 A-E2: Effect of NF-κB and Raf kinase inhibition on lipogenesis and autophagy.** HepG2 cells were pre-treated with BAY for 3 h, followed by AGE stimulation for 12 h. The amount of FAS, Beclin1 and DRAM1 were determined by semi-quantitative PCR from total RNA extracted from these cells (A). WCE prepared from cells treated with BAY and SR for 3 h and then stimulated with AGE for 12 h. The level of LC3B, Beclin1 and p62 were determined by Western blot (B). Amounts of LC3B, Beclin1 and p62 were determined by Western blot from WCE of novastatin-treated HepG2 cells followed by AGE stimulation (C). HepG2 cells were pre-treated with novastatin (Nov-2 µg/ml), BAY (2 µM) and SR (10 µM) for 3 h followed by AGE (100 µg/ml) stimulation of 12 h. Absorbance of oil red O stained lipid particles were plotted as Mean±S.E.M (D). Fluorescence intensity of MDC stained autophagosomes were represented as fold increase for similar treatment. Data is extrapolated from three independent experiments (D). HepG2 cells were transfected with IlkBα-DN and treated with AGE for 12 h. Oil red O staining was done to assess lipid droplets (E1). Oil red O staining of IlkBα-DN transfected HepG2 cells treated with AGE for 12 h were quantified for the absorbance of oil red O stained lipid particles and data is represented from three independent experiments (E2). Error bars represent as S.E.M., Student’s t-test * p<0.05 and *** p<0.001.
Furthermore, DNA binding activity of SREBP and NF-κB was determined in BAY and SR pre-treated and AGE stimulated HepG2 cells (Fig.5.2.3F1). Novastatin treatment in presence of AGE also resulted in substantial decrease in SREBP DNA binding activity (Fig.5.2.3F2).

NF-κB repression by *IkBa-DN* transfection decreased AGE-mediated lipogenesis and autophagy. These data confirm that Raf kinase and NF-κB are major contributor in the regulation of AGE-mediated lipogenesis and autophagy. Also, lipogenesis may not induce autophagy as novastatin did not affect autophagy markers however, autophagy inhibition dramatically dropped lipid accumulation level upon AGE stimulation which needs further investigation.

### 5.2.4 Regulation of lipogenesis at transcriptional level

AGE increases NF-κB DNA binding activity. From above data we confirmed the vital role of NF-κB in lipogenesis regulation. SREBP is another transcription factor which gets activated by AGE through RAGE signalling and is involved in lipid metabolism. We hypothesized that AGE induced NF-κB acts as surveillance factor for SREBP targeted signalling in lipogenesis. To investigate this, HepG2 cells were treated with AGE for different time period and gel shift assay was done. SREBP DNA binding was increased upon AGE stimulation in time dependent manner (Fig.5.2.4A). To check the specificity of gel shift assay, SREBP DNA binding activity was examined along with cyclin D1, unlabeled SREBP, SREBP antibody pre-treated nuclear extract and p65. The antibody
pre-treated extract showed specificity of SREBP DNA binding activity (Fig.5.2.4B). To elucidate how AGE activates SREBP, HepG2 cells were treated with brefeldin A (BR), a blocker of vesicular transport of proteins to Golgi apparatus and DNA binding of SREBP was observed. BR suppressed nuclear translocation of SREBP evidently (Fig.5.2.4C). AGE-mediated SREBP increase was not repressed by BR as determined by semi-quantitative PCR (Fig.5.2.4D) however, Western blot analysis showed that BR was able to inhibit AGE mediated activation of SREBP (Fig.5.2.4E). SREBP level was significantly reduced in *IκBα-DN* transfected AGE stimulated HepG2 cells (Fig.5.2.4F). BAY treatment in HepG2 also showed reduction in SREBP DNA binding activity (Fig.5.2.4G) confirming the role of NF-κB in regulating SREBP expression.

![Figure 5.2.4: Role of SREBP in lipogenesis.](image)

HepG2 cells were treated with AGE (100µg/ml) for 0-48 h. SREBP DNA binding activity was checked by electrophoretic mobility shift assay (A). NE from AGE-induced cells incubated with 2 µg of anti-SREBP, -cyclin D1, or -p65 antibody or unlabeled SREBP DNA (200 ng, double stranded) for 1 h at 4°C and SREBP DNA binding was assayed (B). Cells treated with brefeldin A (BR) for 3 h and followed by AGE stimulation for 12 h. SREBP DNA binding was determined from NE (C). Semi-quantitative PCR was done for similar treatment in HepG2 to determine the amount of SREBP mRNA and actin (D). The amount of SREBP was determined from BR pretreated and AGE-stimulated cells by Western blot (E). HepG2 cells transfected with *IκBα-DN* and treated with AGE for 12 h was assayed for SREBP expression by Western blot (F). SREBP DNA binding was analyzed by gel electrophoresis in HepG2 cells treated with BAY in presence or absence of AGE (G).
Collectively, these results suggest that AGE activates SREBP at transcriptional level rather than regulating the protein processing in Golgi bodies. Also, NF-κB acts as main controller in AGE mediated lipogenesis by regulating the SREBP.

5.2.5 Role of autophagy in lipogenesis

Earlier we showed decrease in lipogenesis by autophagy inhibitors (Fig.5.2.1C). To investigate the role of autophagy in lipogenesis, we knocked down autophagy genes ATG7 and ATG12 in HepG2 cells and assessed the effect of AGE stimulation in these cells by oil red O staining and its absorbance (Fig.5.2.5A and Fig.5.2.5B). We observed less lipids in ATG7 and ATG12 knocked down cells but, AGE stimulation in these cells did not show significant reduction in lipid droplets. AGE stimulation was able to compromise the inhibitory block created by autophagy inhibition. This data indicate that AGE stimulation abrogates effects of autophagy blockage, leading to lipid accumulation.

![Figure 5.2.5 A & B: Role of autophagy in lipogenesis.](image)

Further, AGE mediated autophagy and lipogenesis were compared to classical lipogenesis inducer glucose. AGE induced lipogenesis was significantly very high from glucose-mediated lipogenesis. Wortmannin and rapamycin had no effect on lipogenesis induction (Fig.5.2.5C). Rapamycin increased autophagy but not lipogenesis and wortmannin and novastatin did not increase autophagy or lipogenesis. Caffeine or EGCG
(epigallocatechin gallate), the lypolytic activators, caused substantial reduction in lipid accumulation (Fig.5.2.5C and Fig.5.2.5D). Novastatin reduced both glucose and AGE-mediated lipogenesis but had no effect on autophagy (Fig.5.2.5E).

Figure 5.2.5 C-E: Effect of various lipogenic and lipolysis activators on lipid accumulation. HepG2 cells were stimulated with AGE (100 µg/ml), glucose (25 mM), RAP (100 nM), WM (100 nM), Nov (2 µg/ml), epigallocatechin 3-gallate (EGCG, 10 µM) and caffeine (0.5 µM) for 12 h followed by oil Red O staining. Images were taken and represented (C). HepG2 cells treated with AGE, RAP, glucose, WM, Nov, EGCG and caffeine for 12 h were subjected to absorption of oil Red O accumulated in cells at 500 nm and fluorescence photometry to determine autophagy index at 525 nm. HepG2 cells were stimulated with AGE and glucose in presence or absence of Nov for 12 h and lipid accumulation as well as autophagy index were determined and represented as mean ± S.E.M. from triplicate samples of two independent experiment (E). Error bars represent as S.E.M., Student’s t-test ‘ns’ indicates not significant, ** p<0.01 and *** p<0.001.
Glucose increased lipogenesis and autophagy however, it was substantially less than the AGE mediated induction as observed in oil red O staining (Fig. 5.2.5F1 and Fig. 5.2.5F2). And, rapamycin treatment did not result in lipid accumulation. Caffeine on the other hand, induced lipolysis and hence, no lipid accumulation was observed (Fig. 5.2.5F1). HepG2 cells were incubated with 25 mM glucose or 100 µg/ml AGE. Glucose treatment showed accumulation of lipid droplets prior to autophagy as observed by dual staining of MDC and oil red O though lipid accumulation was preceded by autophagy in AGE treatment (Fig. 5.2.5F3). These data further suggest that AGE mediated autophagy is independent of lipogenesis but high glucose-mediated lipid accumulation might be activating autophagy.

Figure 5.2.5 F1 & F2: Effect of glucose and AGE on lipogenesis and autophagy. HepG2 cells were treated with glucose, rapamycin, caffeine or AGE separately and then these were subjected to dual staining. First, cells were stained with MDC followed by Oil Red O. Represented images were captured in the same view field (F1). Absorbance of oil red O stained lipid droplets and fluorescence intensity of MDC stained autophagosomes were represented as fold increase from triplicate samples as Mean ± S.D.
5.3 Discussion

Aggressive accumulation of advanced glycation end products (AGE) has been witnessed in hyperglycemic conditions especially in ageing group patients. High glucose often results in disturbed cholesterol metabolism and homeostasis leading to lipid accumulation and thus the rise of obesity problems. There are several reagents with anti-oxidant properties which are used to counteract hyperglycemic problems however, most of them also result in cholesterol rise as side effects in patients. One such example is PPARγ analog glitazone. AGE is known to induce reactive oxygen species however, there is still no evidence of AGE proteins being redox itself. Autophagy is the cellular phenomenon of self-consumption which is devised for maintaining cellular homeostasis by degrading any toxic proteins, lipids or injured organelles. It is beneficial to cells as it recycles back simple amino acids and energy molecules and thus helps cells glide through stresses like starvation. This is why it is called a pro-survival mechanism.
In this section, we illustrated the role of AGE in inducing autophagy and lipogenesis through activation of transcription factors like NF-κB, SREBP and the regulatory mechanism in detail. AGE-RAGE interaction results in activation of several kinases which are pre-dominantly redox-sensitive like ERK, IKK etc. We also demonstrated the involvement of NF-κB as master regulator of lipogenesis through the complex regulation of Raf kinase and MAPK pathway. AGE also increased SREBP DNA binding which is primarily involved in lipid metabolic pathway as an inducing factor. We also found that AGE increased FAS expression and ERK phosphorylation, an important factor required for lipogenesis and SREBP maturation. We showed how AGE mediated SREBP activation is at transcriptional level and AGE is not involved in its processing at Golgi apparatus as brefeldin A, a vesicular transport blocker reduced SREBP cleavage to active form and its translocation to nucleus in presence of AGE. Partial inhibition of signalling kinases ERK and p38 MAPK was achieved through inhibition of Raf kinase or PKC or NF-κB but complete inhibition was observed with NF-κB and Raf kinase inhibitors (BAY and SR) together. NF-κB and Raf kinase inhibitors together were able to significantly reducing the lipid accumulation which suggest they are the chief players in this regulation. Though, AGE-mediated autophagy is meant to clear surplus lipids, we did not find any mechanistic correlation between these two processes. Caffeine and EGCG, both lipolytic activators were able to degrade lipids even in presence of AGE and autophagy inhibitors were found to dramatically drop the lipid accumulation but novastatin, a HMG-CoA inhibitor was not able to reduce autophagy index suggesting that autophagy is not dependent upon lipogenesis though lipogenesis might be getting energy supplements through autophagy channel for its sustenance. Comparison with glucose mediated autophagy and lipogenesis reveals that both inducers might be following different regulatory pathways as novastatin was able to affect lipogenesis in both processes in glucose but novastatin did not reduce AGE-mediated autophagy. Also, glucose mediated lipogenesis seems to start first followed by autophagy but AGE mediated lipogenesis is preceded by autophagy which strongly suggest that autophagy is involved in providing energy to the lipogenesis machinery to proceed and is not involved directly in the generation process itself. AGE mediated lipogenesis predominates the clearance of these lipids by AGE mediated autophagy. The balance perhaps gets shifted to lipogenesis due to ample availability of resources and energy supplements provided by autophagy.
machinery that the clearance function of AGE-induced autophagy appears to be short hand.

Understanding how AGE-mediated autophagy is affecting lipid accumulation will guide us to design new therapeutics to combat the side effects of diabetes and obesity control.

**Figure 5.3:** Model proposed to depict the signalling mechanism of autophagy and lipogenesis induced by AGE.