The Role of Inflammation in Type-2 Diabetes Mellitus and its Correlation with Endothelial dysfunction

Thesis submitted in partial fulfillment of the requirements of

Five year BS-MS Dual Degree Program

Indian Institute of Science Education and Research, Pune

By

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20081024

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DECLARATION

I hereby declare that the content of the thesis entitled "T**he Role of Inflammation in Type-2 Diabetes Mellitus and its Correlation with Endothelial Dysfunction"** is the results of the investigations carried out by me at the Immunology lab, Unilever R&D, Bangalore under the supervision of **Dr. Gautam Banerjee** and the same has not been submitted elsewhere for any other degree.

Ankita Jha

BS-MS Dual degree student

IISER, Pune

CERTIFICATE

This is to certify that this dissertation entitled "**The Role of Inflammation in Type-2 Diabetes Mellitus and its Correlation with Endothelial dysfunction"** towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by **Ms. Ankita Jha,** at **Unilever R&D, Bangalore** under my supervision.

Dr. Gautam Banerjee

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Chapter 1: Introduction

1.1. Type-2 Diabetes Mellitus (T2DM)

1.1.1. Prevalence of Type-2 Diabetes Mellitus

The prevalence rate of metabolic syndrome (obesity, diabetes and cardiovascular diseases) is increasing rapidly in most part of the globe. This has gained more attention in developing and emerging (D & E) countries compared to developed countries (Scully 2012). In developing countries, approximately 50% of the cases remain undiagnosed as reported by World Health Organization (WHO).

Such increase has been attributed to shift in dietary habits, genetic pre-disposition, stress and lifestyle modification. Increase in urbanization, improvement in economic conditions, westernization have led to diets rich in saturated fats, high carbohydrate content and less fiber diet which is coupled with a sedentary lifestyle. This shift in lifestyle has led to increased incidence of metabolic syndrome in these populations as depicted in literature (Misra and Khurana 2008). This growing pandemic of metabolic syndrome thus requires attention and more understanding for prevention and improved early detection. In India and China alone in 2010, the number of people suffering with type 2 diabetes mellitus amount to 170million. By 2030, this number is projected to increase to 200 million. Hence in this study, we have focused on understanding type-2 diabetes mellitus (T2DM) and its association with another important disease of the metabolic syndrome that is cardiovascular disease.

1.1.2. Clinical classification of T2DM and glycemic indices

Type-2 diabetes mellitus is defined as a hyperglycemic condition which is a result of the combination of insulin resistance and progressive loss of pancreatic β-cell. Lifestyle factors like diet and low physical activity are associated with increased energy load on

the physiology of human body (Mogensen et al. 2011) and this increase in energy load increases circulating levels of glucose and fatty acids in blood.

American Diabetic Association (ADA) has defined different glycemic indices and has categorized the population clinically into four groups, namely normal glucose tolerance (NGT, healthy), impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and diabetic population. IFG and IGT are together defined as the pre-diabetic condition (Lu 2009).

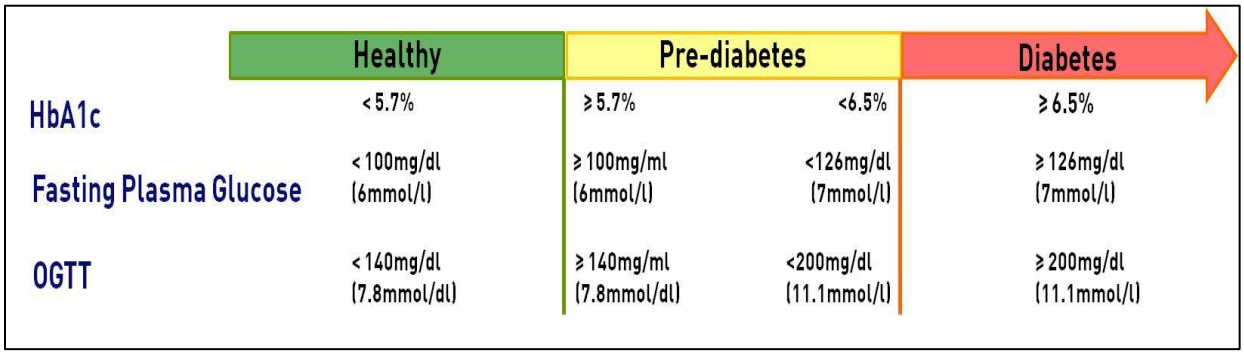

Figure 1: Clinical categorization of population into normal, pre-diabetic and diabetic based on glycemic indices.

1.2. T2DM etiologies and molecular mechanisms

1.2.1. Insulin and glucose homeostasis

Insulin is secreted by the beta cells of the pancreas in response to glucose in blood. Insulin promotes glucose uptake by glucose transporter GLUT4 and controls its utilization by the cells of liver, adipose tissue and skeletal muscles which determines the circulating levels of glucose, free fatty acids and other derivatives in blood and maintains glucose homeostasis (Serdy et al. 2004). Insulin suppresses further addition of glucose to circulation by inhibiting hepatic glucose production and stimulates storage of lipids by adipose tissue. Normal glucose metabolism and sensitivity of cells to insulin are majorly affected in T2DM resulting in reduced insulin stimulated glucose uptake in

periphery, increased hepatic glucose output and increased levels of free fatty acids in circulation (Olefsky and Glass 2010).

1.2.2. Pathways of Glucose metabolism

1.2.2. (a) Glucose phosphorylation

Post incorporation of glucose by tissues, it gets phosphorylated by hexokinases. High affinities Hexokinase II is found mostly in insulin responsive skeletal and adipose tissue and thus help in glucose uptake by these tissues. Low affinity Hexokinase II is predominantly found in pancreatic β-cells and liver and is not modulated by the physiological concentration of Glucose-6-phosphate. Glucose phosphatases catalyze the hydrolysis of Glucose-6-phosphate to produce glucose mainly during starvation (Figure 2).

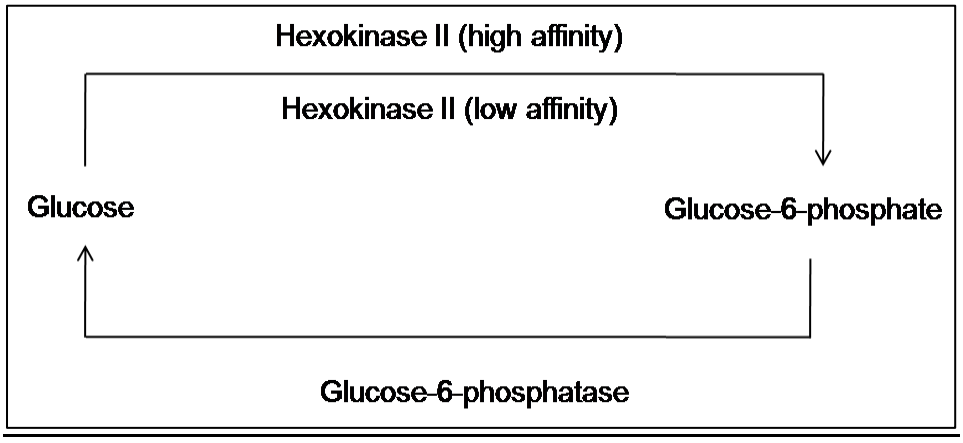

Figure 2: Schematic depicting glucose phosphorylation and enzymes involved.

Hexokinase II has been reported to be low in diabetic patients than healthy individuals and they lack suppression of hepatic glucose production. Low glucose-6-phosphatase

activity has been reported in T2DM patients which leads to increased glucose levels in circulation (Serdy et al. 2004)

1.2.2. (b) Glycolysis and Gluconeogenesis

Glucose 6 phosphate in the cells enters the glycolysis pathway which generates energy by oxidation of glucose into pyruvate or lactate depending on the tissue type. Pyruvate enters the Kreb's cycle and generates ATP as source of energy. Glucose oxidation is significantly impaired and diminished in T2DM patients which in turn impairs insulin signaling as insulin secretion and glycolysis are coupled.

Gluconeogenesis, the process of generating glucose molecules from non carbohydrate sources, mainly amino acids is enhanced in T2DM patients and thus contributes to hyperglycemia in both fasting and post prandial states. In normal individuals, homeostasis is maintained and there is a balance between glycolysis and gluconeogenesis. With loss of this homeostasis as in case of T2DM patients (Figure 2), the other pathways like glycogen synthesis for glucose storage in liver, pentose phosphate pathway for maintaining NADPH and lipid biosynthesis etc are altered and thus have long term effects like increase in free fatty acids in circulation (Serdy et al. 2004)

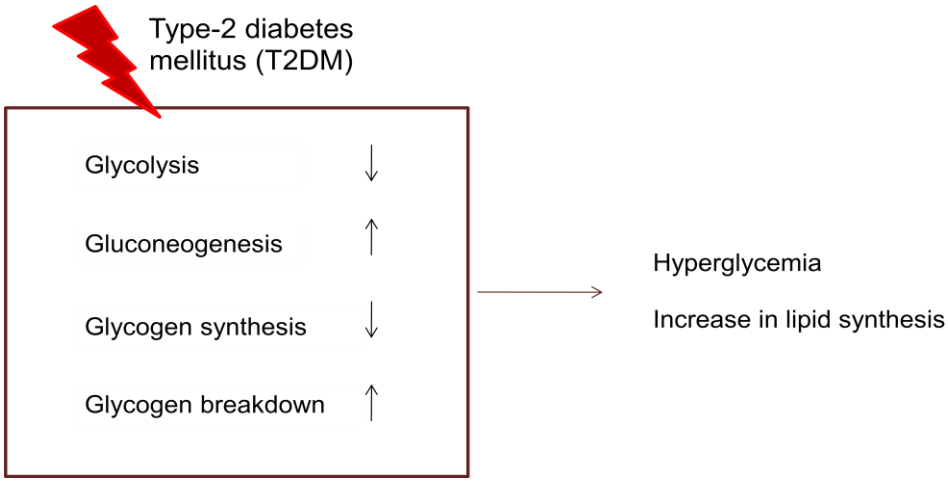

Figure 3: Schematic showing altered glucose metabolism pathways in case of type-2 diabetes mellitus.

1.3. Factors affecting the etiology of T2DM

1.3.1. Adipose tissue function and dysfunction

Adipose tissue is a storage depot for lipids and fatty acids. The lipids from circulation are stored in the form of fat in periods of plenty (post meal) and released to circulation during periods of famine (fasting, increased energy requirements etc). This homeostatic capacity of adipose tissue for the storage of lipids has some limitations. Over-nutrition and increase in the lipid synthesis disturbs this balance and leads to adipose dysfunction and increase in the circulating free fatty acids (FFAs) like palmitate, lineoate etc (Azevedo 2010). These free fatty acids and their derivatives are known to give rise to different patho-physiological conditions.

In addition to storing fats, adipose tissue secretes various bio-active substances like adipokines with a key role in integration of immune system. These adipokines help in normal signaling function. The adipokines released from adipose depots have a direct access to the liver and also have a huge impact on the inflammatory responses. Adipose dysfunction has been reported to be involved in pathogenesis of various diseases through altered immune system (Scarpelleni and Tack 2012). This alteration in the immune system leads to increase in the circulation of various pro-inflammatory cytokines promoting a state of chronic inflammation and thus causing further release of cytokines like tumor necrosis factor-α (TNF-α), interleukins (IL-6) etc which also mediate increased expression of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and E-selectins. (Hu et al., 2000).

1.3.2. Inflammatory cytokines and T2DM

Recent studies have indicated T2DM to be an inflammatory condition (Wellen and Hotamisligil 2005; Donath and Shoelson 2011). One school of thought is that inflammatory pathways have a causal relationship with disorders like obesity, insulin resistance (IR) (Shoelson et al. 2006; Bastard et al. 2006), type-2 diabetes mellitus (T2DM) and endothelial dysfunction as shown in both animal and human studies, however underlying mechanisms are not well understood (Pradhan et al. 2001). It has also been demonstrated that various cytokines such as Leptin, Tumor necrosis factor –α (TNF-α) and more recently interleukins like IL-6 cause insulin resistance directly or indirectly (Loss et al. 2000).

Epidemiological studies have shown elevated circulating levels of various pro-inflammatory cytokines like TNF-α, interleukins (IL-6, IL-1β) etc. in diabetic patients and have been associated with T2DM after taking into consideration factors like age, body mass index (BMI), etc (Spranger et al. 2003). Similarly high levels of acute phase proteins such as C-Reactive protein (CRP), Plasminogen activator inhibitor and fibrinogen have been found in circulation (Donath and Shoelson 2011). Whether inflammation is a cause or effect of diabetes is yet to be concluded though the association of inflammation and diabetes has been observed in many large human studies.

1.3.3. Effect of adipose dysfunction and inflammatory cytokines

Increased levels of pro-inflammatory cytokines are observed in case of diabetes patients and this is attributed to the up regulation of systemic inflammation and adipocyte signaling. TNF-α has been reported to have direct inhibitory effect on insulin receptor substrates (IRS), glucose transport protein (GLUT4) and have indirect effects on glucose metabolism by increasing free fatty acid oxidation, increasing release of other cytokines like IL-6 etc and thus promoting insulin resistance (Hu et al; 2000).

High levels of cytokines also invoke accumulation of free fatty acids and its derivatives like ceramides. From the myriad of the bio-active lipids that accumulate due to adipose dysfunction sphingolipids and ceramides have gained special attention as T2DM

patients have shown high levels of plasma ceramides (Haus et al. 2009). Ceramides are synthesized through different pathways (Chavez and Summers 2012). It can occur from free fatty acids like palmitate after undergoing a series of reaction and with the help of enzymes like serine palmitoyltransferase-2 (SPT-2) (Schmitz-peiffer 2010). Ceramide formation can also occur from sphingolipids with the help of enzyme spingomyelinase function. High levels of cytokines and free fatty acids like palmitate can increase the transcription levels of enzymes like sphigomyelinase, SPT-2 and cerS resulting in increased ceramide accumulation (Erickson et al. 2012). *In vitro* studies have reported that exogenously added ceramides mimic TNF-α induced insulin resistance and also impair insulin stimulated glucose uptake (Teruel et al. 2001). In addition, ceramides have been reported to increase the reactive oxygen species (ROS) in the system due to induced perturbation of electron transport chain in mitochondria (Bikman and Summers 2011). Mitochondrial abnormalities have been seen in patients with insulin resistance and impairment in function either a cause or the consequence of the condition (Chavez and Summers 2012).

1.3.4. Effects of free fatty acid accumulation

Elevated plasma levels of FFAs like palmitate have been reported to show a role in development of insulin resistance (Figure 3) but the mechanism for the same is not clear (Gao et al. 2004). Free fatty acid treatment have shown to increase the expression of iNOS (induced-nitric oxide synthase) in macrophages (Jeon et al. 2012). iNOS have been reported to induce cyclo-oxygenase-2 gene expression (COX-2) that lead to formation of various inflammatory mediators like nitric oxide and PGE_2 via NFκB pathway (Demarchi et al. 2005).

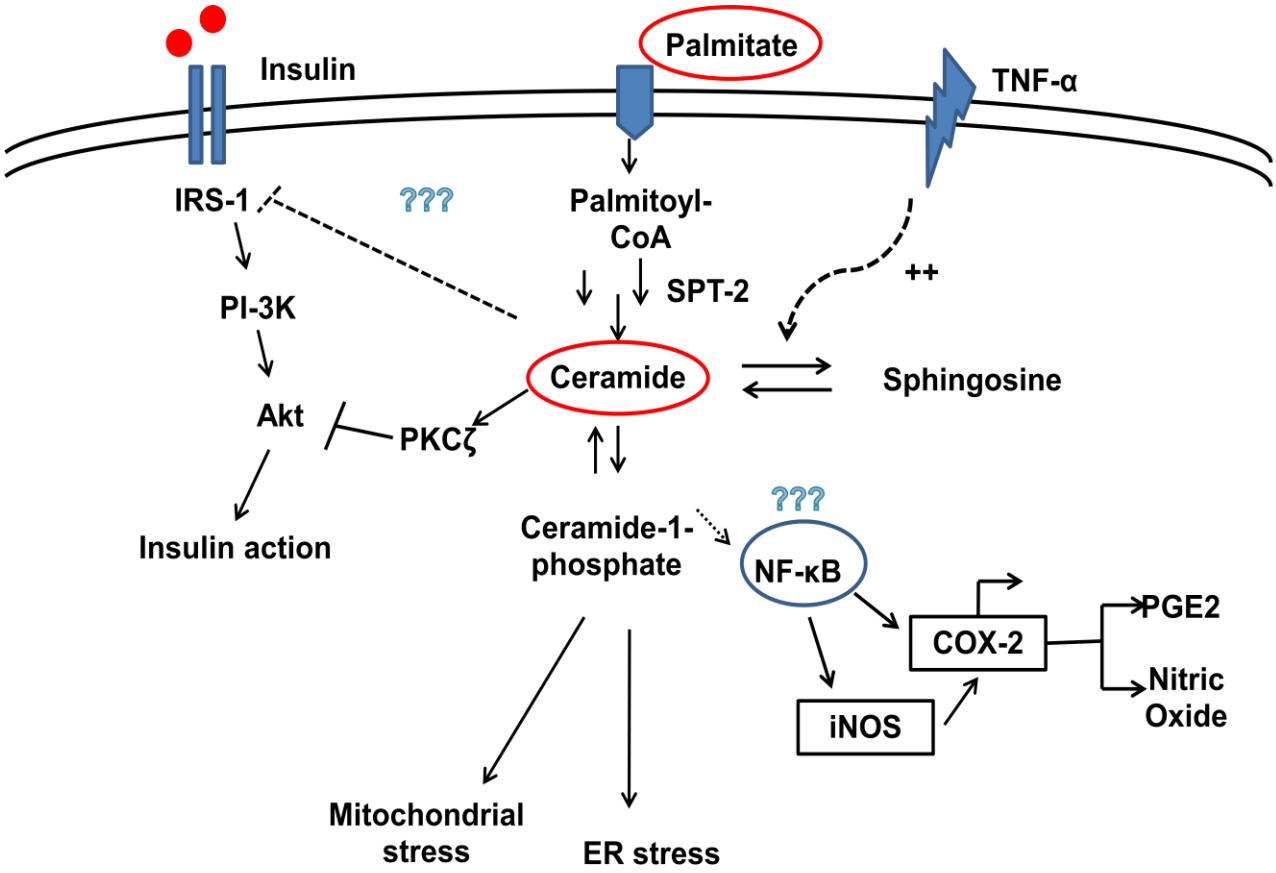

Figure 4: Schematic highlighting the effects of free fatty acids on cross talk between inflammation, insulin resistance and pathways leading to oxidative stress.

1.4. Cardiovascular diseases and its correlation with T2DM

Clinically cardiovascular diseases (CVD) are known to follow type-2 diabetes mellitus (T2DM). There is a close relation between metabolic disturbances, adipose tissue dysfunction, T2DM and CVD (Cascio et al; 2012). A large number of common factors like that of systemic inflammation have been determined to be the common cause of both the problems. As reported high free fatty acids and high inflammatory cytokine levels as in case of T2DM can lead to deleterious effects and thus leads to endothelial dysfunction (Hajer et al; 2008).

1.4.1. Endothelial function

Endothelial homeostasis and normal function is maintained by extracellular matrix and other chemical mediators secreted by endothelium like endothelial- nitric oxide synthase (eNOS) mediated nitric oxide (NO) which have vasodilatory, anti-inflammatory anti-platelet functions, plasminogen activator inhibitor-1 (PAI-1) and other adhesion molecules like VCAM, ICAM (Oever et al. 2010).

1.4.2. Endothelial dysfunction

The endothelial NO generation occurs through insulin dependent pathway that leads to phosphorylation of eNOS in calcium-independent manner (Francis Kim et al. 2005). Current hypothesis suggests that endothelial dysfunction occurs due to decrease in endothelial NO availability as well. This decrease can be due to either NO quenching or loss of NO production. Former is known to occur because of increase in the oxidative stress of the system (generation of reactive oxygen species) and latter has been reported due to increase in inflammation because of free fatty acids (Oever et al. 2010).

Endothelial cells contain two nitric oxide synthase eNOS and iNOS. While eNOS is majorly localized in plasma membrane and golgi of endothelial cells (Balligand et al; 2009; Shaul 2002). iNOS (inducible nitric oxide synthase) is mostly localized in endoplasmic reticulum and perinuclear space. In the presence of inflammatory mediators iNOS releases high levels of nitric oxide in calcium dependent manner and causes deleterious functions like nitrosylation of proteins in endoplasmic reticulum and thus lead to dysfunctions.

In normal healthy individuals, eNOS localises to calveolae (micro domains of plasma membrane) by binding to the calveolin-3 protein. This co localization facilitates protective release of NO. Current hypothesis suggests that

- ceramides are enriched in calveolae
- studies have shown palmitate has an effect on binding of eNOS to calveloae, which in turn might be effecting the localization of eNOS in plasma membrane

and thus changing the phosphorylated status of the enzyme (Knowles et al. 2011; Roszer 2012).

- Serine phosphorylation of proteins like Akt, eNOS have been reported to be inhibited by free fatty acids in circulation but it is not well understood if free fatty acids directly modulate the eNOS function.

Exposing cardiomyocytes to increased levels of lipids like palmitate induces contractile dysfunction.

Objective of the work:

Process of Inflammatory pathway induction by increased levels of free fatty acids like palmitate, ceramides etc is not clearly understood. The objective of this study is

i. To understand the effect of high glucose and free fatty acids on inflammation *in-vitro* specifically evaluating different mediators of inflammation like COX-2 and iNOS-dependent nitric oxide.

ii. To understand the effect of free fatty acids on endothelial cells and mechanism of endothelial dysfunction

Chapter 2: Materials and Methods

2.1. Cell culture

H-4-II-E (rat liver epithelial cells, obtained from ATCC, CRL-2922) and EA.hy926 (human endothelial cells, obtained from ATCC, CRL-1548) were maintained in DMEM high glucose and 10% (w/v) fetal bovine serum (GIBCO). Cells were sub-cultured regularly and maintained at 37^{o}C and 5% CO_2 incubator. Cells were grown to confluence and seeded in 6 well, 24 well plates for 24 hours before treatment.

2.2. Preparation of media containing fatty acid-albumin complexes

Saturated palmitate (SIGMA) and C2-ceramide (SIGMA) were used in the study. Lipid containing media was prepared by conjugation of these free fatty acids to BSA using following method.

2.2.1. Palmitate preparation (1mM stock): 100ml of 150mM NaCl (Sigma was prepared in water. 2mM of palmitate is dissolved in 500µl of ethanol and stirred till it dissolved. This solution was then mixed with 50 ml of 150mM NaCl. 50 ml of 150mM NaCl was used to prepare 2mM fatty acid free BSA (Bovine serum albumin, GIBCO). Both the solutions were mixed and stirred at 70^{o}C.

2.2.2. Ceramide preparation (1mM stock): 100ml of 150mM NaCl (Sigma) was prepared in water. 2mM of palmitate was dissolved in 200mM of DMSO and stirred. This was mixed with 50 ml of 150mM NaCl. 50 ml of 150mM NaCl was used to prepare 2mM of fatty acid free BSA (Bovine serum albumin, GIBCO). Both the solutions were mixed and stirred at70^{o}C.

2.3. Free fatty acid treatment

Working concentration of free fatty acids was prepared in DMEM without fetal bovine serum. Cells were given two washes with 1X PBS and free fatty acid (ceramide and or palmitate) treatment was given for 24 hours at 37^{o}C and 5% CO_2 incubator.

2.4. MTT colorimetric assay

MTT assay was used to determine the viability of cells after treatment. In this assay, yellow MTT (tetrazolium dye) gets reduced to purple formazan in the mitochondria of the living cells and thus viability is quantified.

The media was removed and cells were washed with 1X PBS. 5mg/ml stock MTT reagent (Sigma) was prepared in 1 X PBS. 100µl working MTT reagent (1:10 dilution of stock in 1 X PBS) was added to each well and was incubated for 4 hours. Post incubation, the reagent was discarded and to the cells,100µl of DMSO was added to lyse the cell and the color development was measured in BIORAD microplate reader-MDL 680 at 590nm.

2.5. RNA isolation

Media was removed from the cell culture after treatment. 1ml TRI reagent (Sigma-Aldrich) was added to the cells. Cell lysate was passed through pipette several times and was transferred to micro-centrifuge tubes. The tubes were left at room temperature for 10 min and then stored at -20^{o}C till RNA isolation.

At the time of RNA isolation, 0.3 ml of chloroform was added to each tube and kept at Room Temperature for 20 min after a brief vortex. The mixture was centrifuged at 12,000 RPM for 10 min. The clear aqueous phase was transferred to a fresh tube. 0.5 ml of isopropanol was added to the aqueous phase and kept at RT for 10 min after gentle mixing. Supernatant was removed after centrifugation at 14,000 rpm for 30 min at 4^{o}C. To the pellet, 0.7 ml of 70% ethanol was added. The tube was centrifuged at 14,000 rpm for 30 min. Pellet was air dried and RNA was re-suspended in DEPC treated water.

2.6. cDNA synthesis

RNA purity and amount was quantified using Thermo-scientific Nanodrop spectrophotometer (ND 200C) with Nanodrop 2000c software at A260 and A280. cDNA synthesis was carried out using BIORAD iScript cDNA synthesis kit. A reaction mixture of 20µl was prepared with 4µl of 5X reaction buffer, 1µl of reverse transcriptase enzyme

<1µg RNA as template and nuclease free water.) c-DNA was prepared using the following cycle - 25^{o}C for 5min, 42^{o}C for 30 min, 85^{o}C for 5min and at 4^{o}C for1 hr..

2.7. PCR reaction (Polymerase chain reaction)

Total RNA extracted from tissues using TRI reagent and cDNA was synthesized as mentioned above and stored at -20^{o}C. PCR reaction components (Thermo scientific) were brought to 4^{o}C on ice. Reaction mix of 50µl was prepared using 25µl of PCR Master Mix (2X), forward and reverse primers for COX-2, 1µl of cDNA and the volume was made up with water. PCR reaction was carried out as follows- (1) Initial denaturation was carried out by incubation at 95^{o}C for 5 min (2) subsequent denaturation was carried out for 45 sec at 95^{o}C (3) annealing was done at 56^{o}C for 45 sec (4) extension at 72^{o}C for 45 sec (5) final extension for 5min at 72^{o}C was carried out and stored at 4^{o}C. Steps (2) to (4) was repeated 28 times for COX-2. Entire PCR cycle was repeated for 24 cycles for β-actin with beta actin primers in place of Cox 2 primers. The data was normalized to the levels of β-actin expression.

2.8. PCR sample analysis

All PCR samples were analyzed on 0.8% agarose gels containing ethidium bromide. 4µl of 6X loading dye was added to each sample and was run in 1X TAE buffer at 100V. Standard DNA ladder was run along with the samples. Gel pictures were taken using Gel documentation system equipped with Scion zoom camera and 1DSCAN Ex (Scanalytics.In). Analysis was done using ImageJ 1.34s software with gel analysis tool.

2.9. Immuno-staining

EA.hy926 human endothelial cells were grown on sterile glass coverslips overnight at 37^{o}C and 5% CO_2. Cells were then treated with different concentration (10µM, 5µM and 1µM) of Palmitoyl-coA NBD FITC conjugated (AVANTI) prepared in DMEM high glucose media without fetal bovine serum. After 16 hrs the supernatant was removed and cells were washed with 1X PBS buffer. Cells were fixed with 4% para-formaldehyde for 10 min. Primary antibody staining was carried out with anti eNOS (Sigma) antibody tagged with TRITC (Sigma-Aldrich) and secondary anti-rabbit antibody tagged with DAPI.

Images were captured using 100X oil immersion of Olympus BX40 inverted epi-flurorescence microscope equipped with ImagePro Express 6.3.

2.10. Determination of Nitric oxide and Reactive oxygen species using flow cytometry

FACS (Fluorescence activated cell sorting) - flow cytometry was performed to detect nitric oxide (NO) and reactive oxygen species (ROS) after treatment with free fatty acids. Measurement of intracellular NO production was carried out using Nitric oxide-sensitive fluorescent dye, DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) from Invitrogen with increased fluorescence post formation of NO_2^- from NO with λ_{EX} and λ_{EM} at 488nm and 520nm respectively. ROS levels were monitored using a reagent DCFDA (2',7'-dichlorofluorescein diacetate) a fluorogenic dye (Invitrogen) that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the cell with λ_{EX} and λ_{EM} at 495nm and 529nm respectively. Cells were treated with free fatty acids or with inhibitors LNAME (N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride) (Invitrogen) and 1400W (N-(3-(Aminomethyl)benzyl)acetamidine) (Invitrogen) for 24 hrs and were incubated with DAF-FM and DCFDA (1:1000) in DMEM- high glucose without FBS for 45 minutes. Inhibitors were directly added to DMEM without FBS at the required concentration. In experiments with eNOS agonist treatment, cells were incubated for 45 minutes in eNOS stimulator and then the dye was after the removal of the supernatant. Cells were washed with EBSS (Earle's Balanced Salt Solution by GIBCO) and fixed with 4% para-formaldehyde solution after trypsinization with 0.25% trypsin in 0.25mM EDTA solution. Cell suspension was transferred to 96-well microtiter plate and analysed by Flow cytometer (BD FACS Callibur) with dual lazer at 480 nm and software Cell Quest Pro.

Chapter 3: Results

3.1. Effect of high glucose on hepatocytes

3.1.1. Characterization of dose dependent viability of glucose in H4IIE (rat hepatocytes)

H-4-II-E, rat hepatocytes have been extensively used to study glucose metabolism. MTT assay was done to understand the effect of high glucose *in vitro* (as described in 2.4). The graph (Figure 5) below describes the viability of rat hepatocytes in presence of different concentrations of glucose.

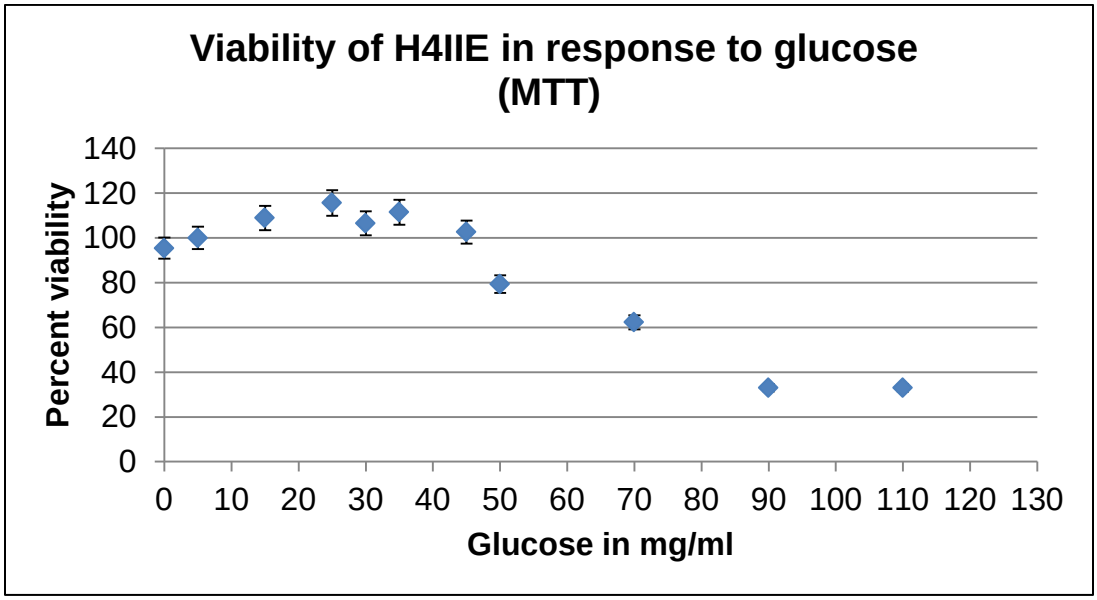

Figure 5: Dose dependent effects of glucose (5mg/ml to 110mg/ml) on viability of H4IIE cells. Results shown as mean ± SD.

Treatment with normal glucose (5mg/ml) did not cause cell death (95% - 99% viability). The viability of the hepatocytes started declining at 50 mg/ml (80% viability) and at 100mg/ml only 30% of the cells were viable.

3.1.2. Effect of high glucose on COX-2 expression

Cyclooxgenase-2 (COX-2) has been reported to be readily induced in variety of cells by inflammatory cytokines and other stimuli. H4IIE rat hepatocytes were treated with sub-toxic glucose levels (50mg/ml and 25mg/ml) for 4 hrs and 24 hrs. RNA was isolated from hepatocytes (as described in 2.5) post treatment and PCR was performed (method described in 2.6 and 2.7) to evaluate the effect of treatment on expression of COX-2. The data was normalized with the expression of β-actin, a housekeeping gene. Expression levels of COX-2 were quantified by the intensity analysis from the images of the bands with the help of ImageJ and the results were reported as ratio of COX-2/ beta-actin (Figure 6 and 7).

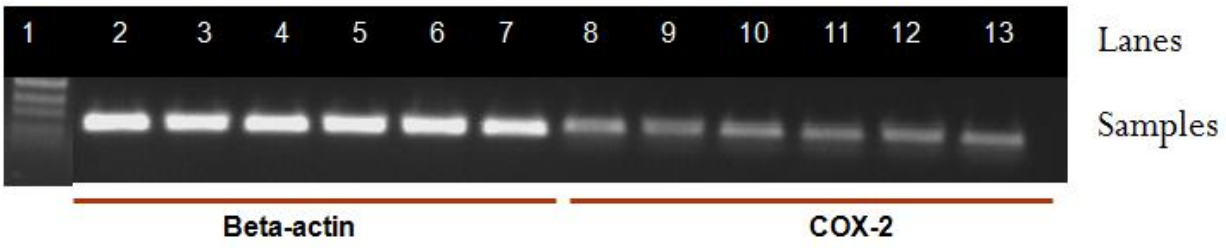

Figure 6: Expression levels of COX-2 and β-actin in hepatocytes treated with different concentrations of glucose (5 mg/ml, 25 mg/ml and 50mg/ml) for 4 hrs and 24 hrs. Details of each lane and samples run are in table 1.

Lane Number	Sample	Gene expression
1	Ladder	
2	Control (5mg/ml) + 24 hrs	Beta- actin
3	Glucose 25 mg/ml +24 hrs	Beta- actin
4	Glucose 50 mg/ml + 24hrs	Beta- actin
5	Control (5mg/ml) + 4 hrs	Beta- actin
6	Glucose 25 mg/ml +4 hrs	Beta- actin
7	Glucose 50 mg/ml + 4hrs	Beta- actin
8	Control (5mg/ml) + 24 hrs	COX-2
9	Glucose 25 mg/ml +24 hrs	COX-2
10	Glucose 50 mg/ml + 24hrs	COX-2
11	Control (5mg/ml) + 4 hrs	COX-2
12	Glucose 25 mg/ml +4 hrs	COX-2
13	Glucose 50 mg/ml + 4hrs	COX-2

Table 1: Depicts different PCR samples being run corresponding to the lane number in figure 5 for COX-2 and beta-actin expression levels after treatment with different concentration of glucose on hepatocytes for 4 hrs and 24 hrs.

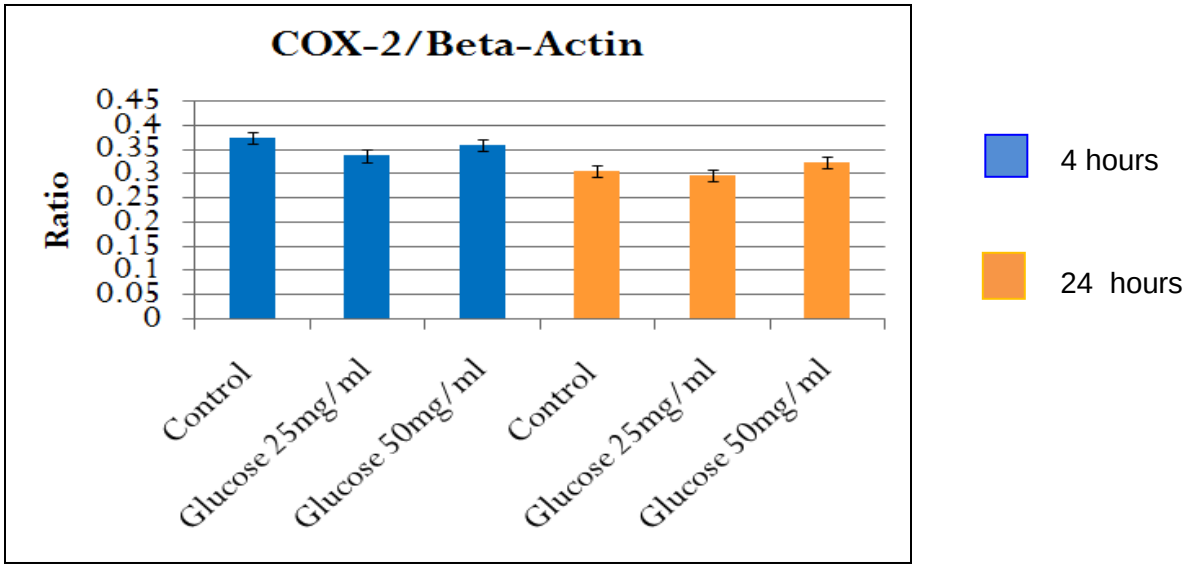

Figure 7: Ratio of COX-2 / beta-actin expression levels after treatment with different concentration glucose on hepatocytes for 4hrs and 24 hrs.

There was no significant change in the expression levels of COX-2 in H4IIE rat hepatocytes post treatment with different concentrations (5 mg/ml, 25 mg/ml and 50mg/ml) of glucose for 4 hrs and 24 hrs (Figure 7).

3.2. Effect of free fatty acids on hepatocytes

3.2.1. Effect of C2-ceramide on COX-2 expression

In order to determine the effect of free fatty acids on inflammatory pathways, hepatocytes were treated with sub toxic levels (3.125µM to 50µM) of C2-ceramides for 4 hrs and 24 hrs and COX-2 expression levels were evaluated. RNA was isolated from hepatocytes after treatment (method described in 2.5) and PCR was performed (as described in 2.7) to evaluate the changes in expression levels of COX-2 which was normalized with the expression levels of β-actin. Expression of COX-2 was quantified from intensity of the bands of the PCR samples run on agarose gel (Figure 8) with the help of ImageJ and were reported as ratio of COX-2 expression/ beta-actin expression (Figure 9).

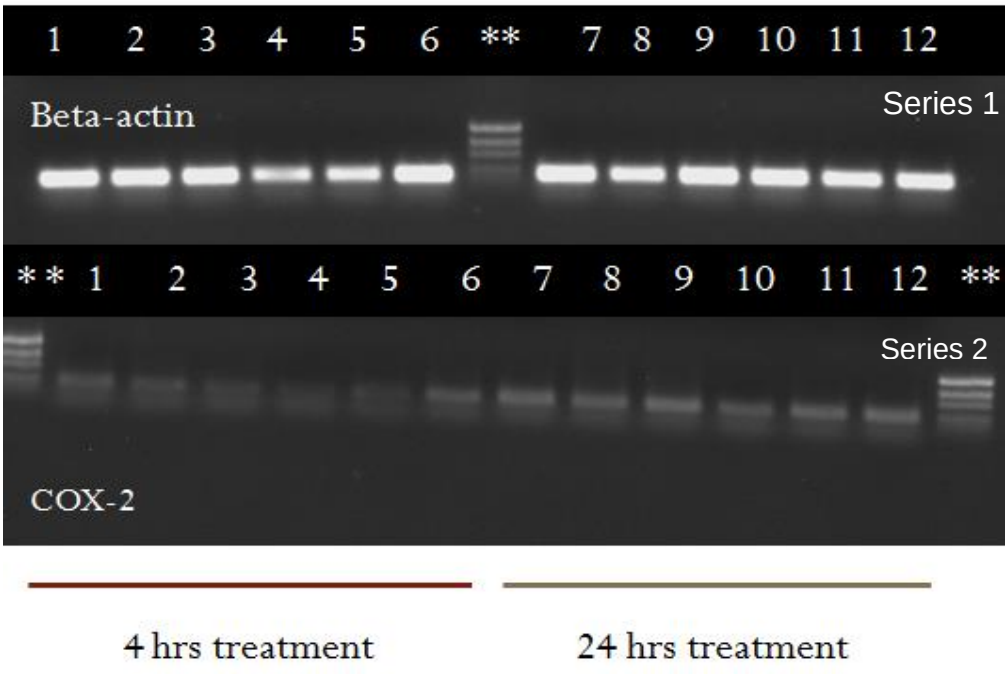

Figure 8: Expression levels of COX-2 and beta-actin after PCR from different concentrations of ceramide treated samples for 4 hrs and 24 hrs.

Lane number	Samples
**	Ladder
1	Control (no ceramide) + 4hrs
2	Ceramide 50µM + 4 hrs
3	Ceramide 25µM + 4 hrs
4	Ceramide 12.5µM + 4 hrs
5	Ceramide 6.25µM + 4 hrs
6	Ceramide 3.125µM + 4 hrs
7	Control (no ceramide) + 24hrs
8	Ceramide 50µM + 24 hrs
9	Ceramide 25µM + 24 hrs
10	Ceramide 12.5µM + 24 hrs
11	Ceramide 6.25µM + 24 hrs
12	Ceramide 3.125µM + 24 hrs

Table 2: Depicts different samples being run corresponding to the lane number in figure 7 for COX-2 (series2) and beta-actin (series1) expression levels after treatment with different concentration of ceramides on hepatocytes for 4hrs and 24 hrs.

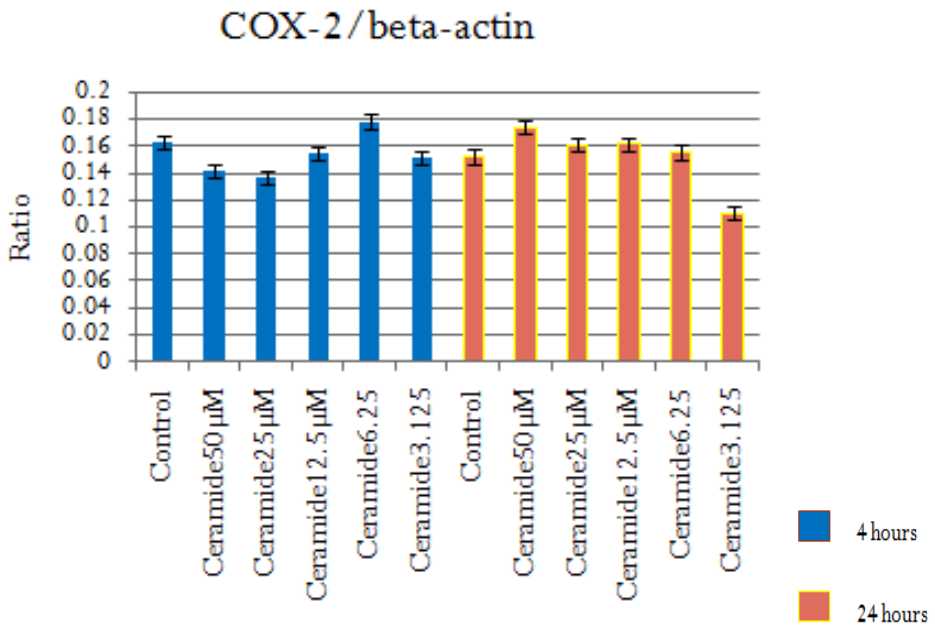

Figure 9: Ratio of COX-2 / beta-actin expression levels after treatment with different concentration of C2-ceramides on hepatocytes for 4hrs and 24 hrs.

There was no significant change in the expression levels of COX-2 after H4IIE rat hepatocytes were treated with different sub-toxic concentrations of C2-ceramide.

3.2.2. C2-ceramide treatment and levels of nitric oxide in hepatocytes

In order to determine the induction of inflammatory pathways in response to C2-ceramide, H4IIE rat hepatocytes were treated with different sub-toxic concentrations of C2-ceramides (5µM, 25µM) as (described in 2.3). After 24 hours of treatment, nitric oxide levels were quantified using flow cytometry (Figure 11) as described earlier (2.10). The results are reported as fold change of mean intensity values with respect to the untreated control (Figure 10).

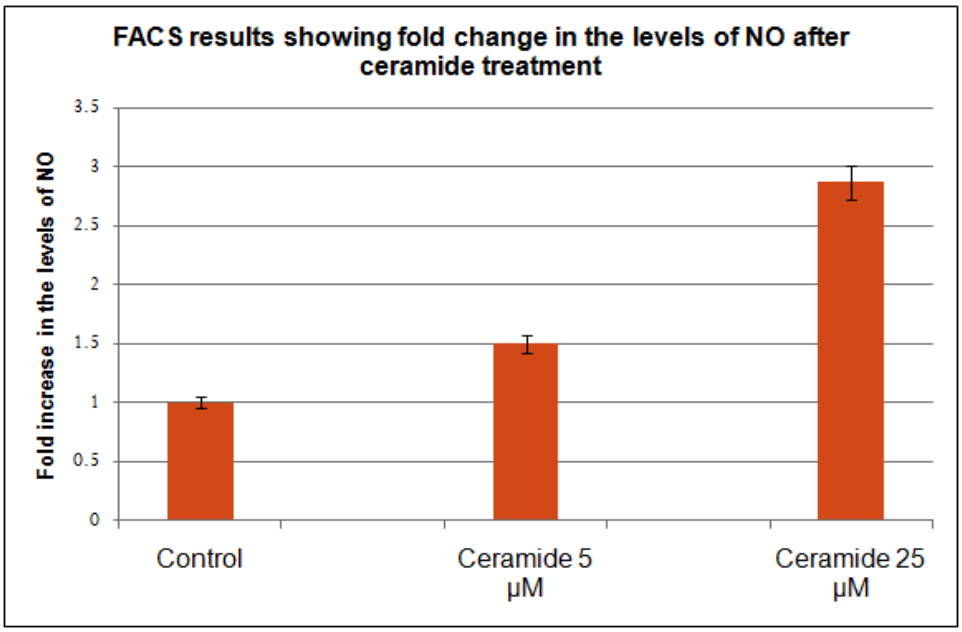

Figure 10: Fold increase in the levels of nitric oxide after treatment with different concentration of C2-ceramide (5µM, 25µM) with respect to the control (without treatment). Data has been reported as Mean ± SD.

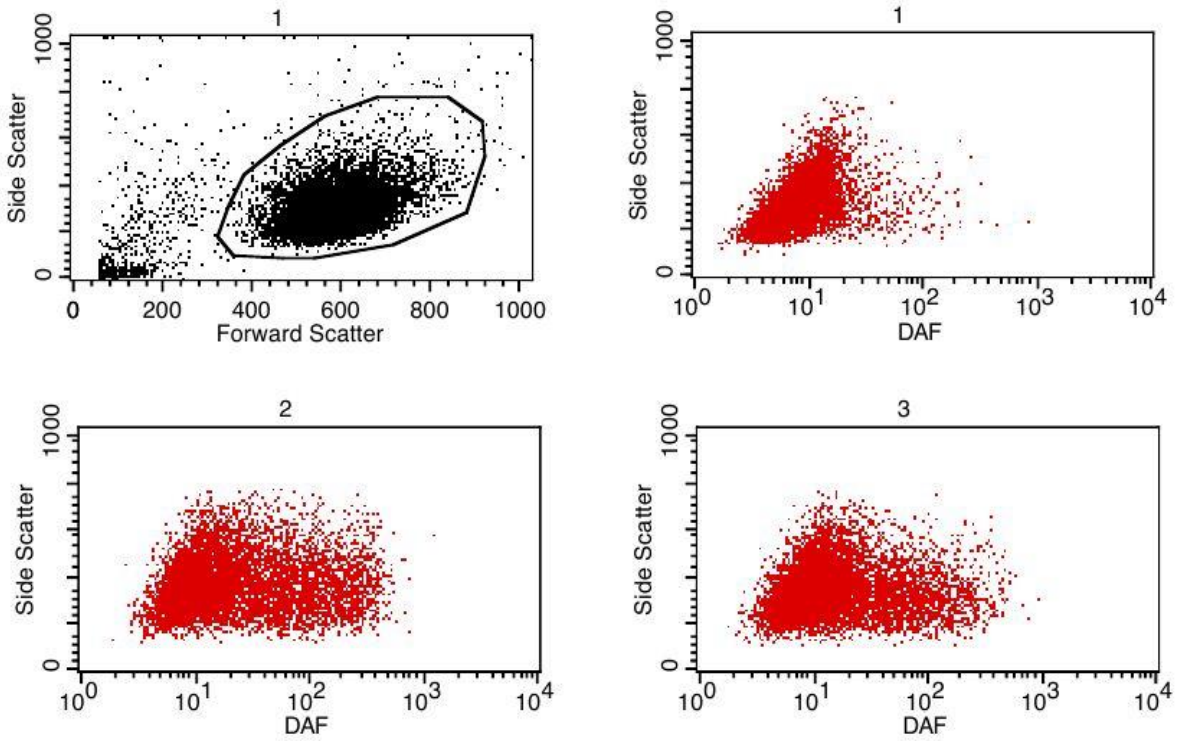

Figure 11: Scatter plots showing FACS results after C2-ceramide treatment in H4IIE rat hepatocytes. Black scatter depicts the population selected for flow cytometry. Red scatter shows fluorescence intensity due to DAF that has bound to the nitric oxide present in cells. An increase in the fluorescence scatter was observed after C2- ceramide treatment (scatter plot 2- 25μM ceramide treatment, scatter plot 1-5μM ceramide treatment) with respect to control (scatter plot 1).

The C2-ceramide treatment in hepatocytes did not induce COX-2 expression but there was a significant increase in the levels of nitric oxide suggesting induction of inflammatory pathways. Similar results were obtained after palmitate treatment on H4IIE rat hepatocytes. There was a significant increase in the levels of NO (data not shown).

3.3. Effect of free fatty acids on endothelial cells

3.3.1. C2-ceramide treatment and levels of nitric oxide

To evaluate the response of endothelial cells to C2-ceramide, the cells were treated with different sub-toxic concentrations of C2-ceramides (5µM, 1µM) as described (2.3). After 24 hours of treatment, DAF was added for nitric oxide detection. Cells were treated with or without eNOS stimulator agonist as indicated for 45 minutes (5µM). Nitric oxide levels were determined using flow cytometry (as described in 2.10) and were reported as fold change of mean intensity values with respect to the untreated control (Figure 12).

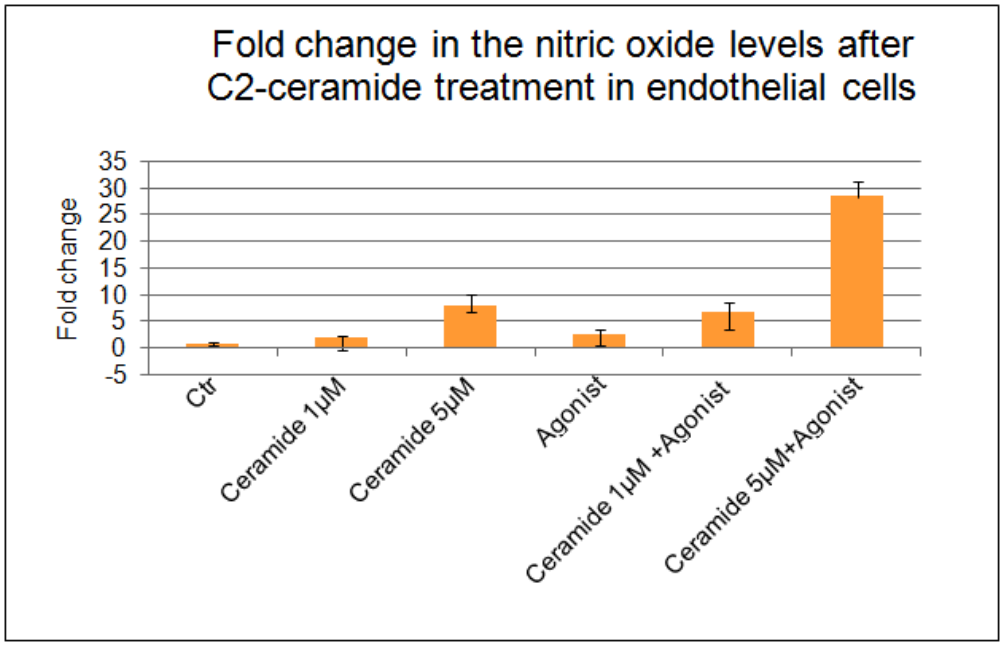

Figure 12: Fold increase in the levels of nitric oxide after treatment with different concentration of C2-ceramide (5µM, 1µM) with respect to the control (without treatment) in endothelial cells. Cells were also treated with 5µM of eNOS stimulator as positive control. Data has been reported as mean ± SD .

Significant increase in the levels of nitric oxide was seen after ceramide treatment with respect to untreated control. This indicates that free fatty acid derivatives like ceramide induce nitric oxide dependent inflammatory response.

3.3.2. Palmitate treatment and levels of nitric oxide

Endothelial cells were treated with different concentrations of palmitate to evaluate their response to free fatty acids. Endothelial cells were treated with palmitate (100µM, 200µM) for 24 hours (described in 2.3) and levels of nitric oxide were quantified after addition of DAF and eNOS stimulator (5µM) agonist as a positive control (described in 2.10). Fold change in the mean intensity of flow cytometry after treatment was reported (Figure 13).

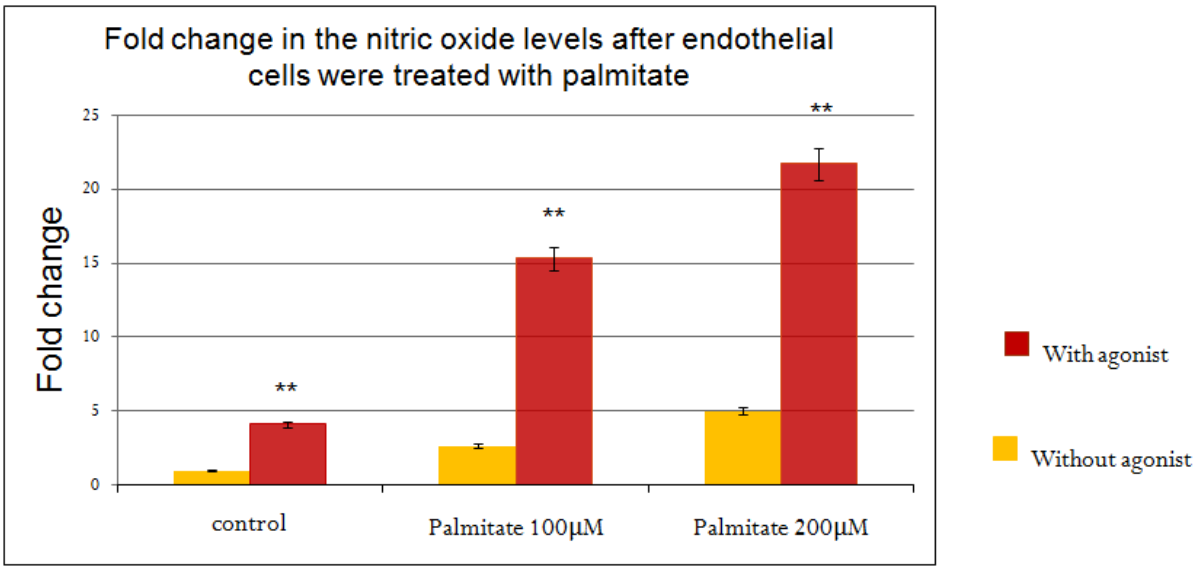

Figure 13: Fold change in the levels of nitric oxide after treatment with different concentrations of palmitate (100µM, 200µM) with respect to the control in endothelial cells. Cells were also treated with 5µM of eNOS stimulator as positive control. Bars with ** show agonist treatment. Data has been reported as mean ± SD values.

Increase in the levels of nitric oxide was observed after palmitate treatment (with both with and without agonist treatment). Similar observations were made in case of ceramide treatment. With eNOS stimulator and palmitate treatment, a huge increase in

the levels of nitric oxide was observed which suggested the role of these free fatty acids in modulating eNOS and iNOS.

3.3.3. Effect of LNAME (NOS inhibitor) on palmitate induced response

To understand the effect of free fatty acids on the iNOS and eNOS modulation, endothelial cells were incubated with palmitate (100µM, 200µM) or palmitate + LNAME (non specific NOS inhibitor, 1mM) for 24 hours (described in 2.10). Nitric oxide levels were measured after sequential addition of DAF and eNOS stimulator incubated for 45 min each. Arrows indicate LNAME (1mM) treatment on control, palmitate treated cells and eNOS stimulated cells (Figure 14).

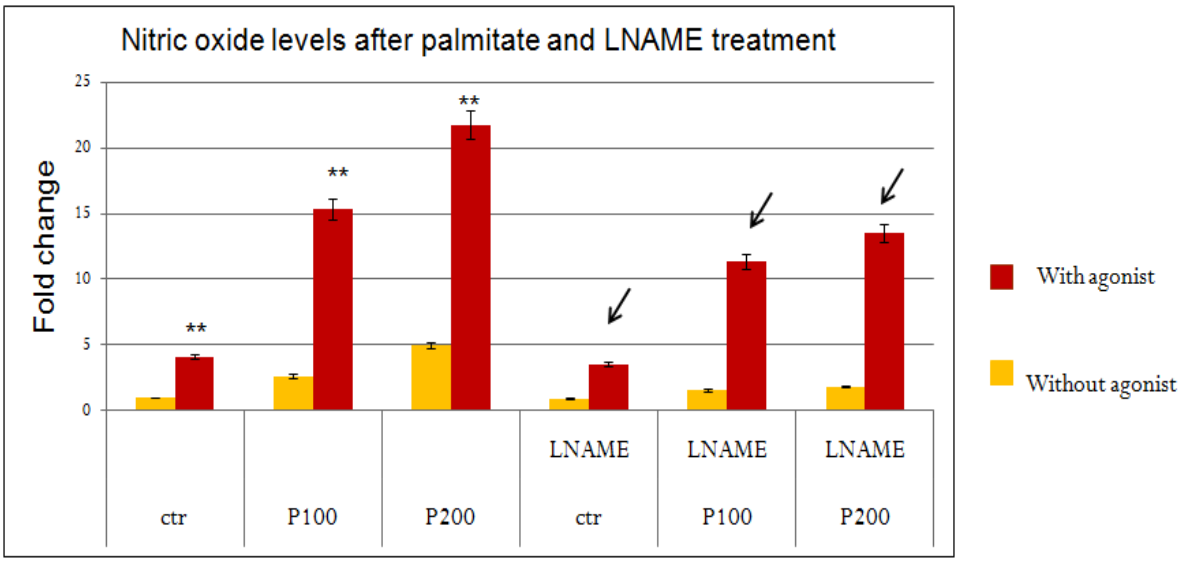

Figure 14: Fold increase in the levels of nitric oxide after treatment with different concentration of palmitate (100µM, 200µM) with respect to the control (without treatment) in endothelial cells. Cells were also treated with 5µM of eNOS stimulator as positive control. Bars with ** show agonist treatment. . Data has been reported as Mean ± SD.

LNAME is a non specific NOS inhibitor and thus cells treated with LNAME showed a decrease in the levels of nitric oxide. There was an increase in the levels of nitric oxide after palmitate treatment (without eNOS stimulator), which almost came down to the

levels of control. In case where cells were treated with eNOS stimulator, LNAME treatment brought down the levels of nitric oxide. Data suggests that LNAME might be acting on one or both the enzymes, eNOS and iNOS to bring down the levels of nitric oxide.

3.3.4. Effect of 1400W (iNOS inhibitor) on palmitate induced response

To understand the effect of free fatty acids on the iNOS and eNOS modulation endothelial cells were pre-incubated with palmitate (100µM, 200µM) or palmitate + 1400W (iNOS inhibitor, 20nM) for 24 hours and nitric oxide levels were measured after sequential addition of DAF and eNOS stimulator and incubated for 45 min each (as described in 2.10).

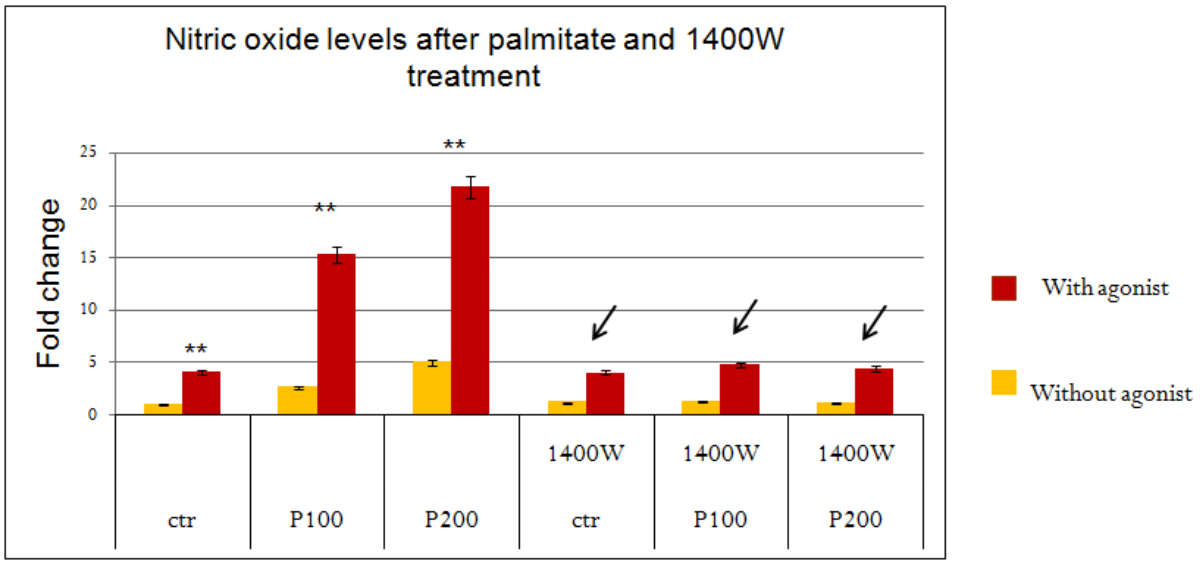

Figure 15: Fold change in the levels of nitric oxide in endothelial cells after treatment different concentration of palmitate (100µM, 200µM) with respect to the control (without treatment). Cells were also treated with 5µM of eNOS stimulator as positive control. Bars with ** show agonist treatment. Arrows indicate 1400W (20nM) treatment on control, palmitate treated cells and eNOS stimulated cells. Data has been reported as Mean ± SD.

Treatment with 1400W (iNOS) inhibitor brought down the levels of nitric oxide. In the cells not treated with eNOS stimulator, 1400W treatment decreased the levels palmitate

induced nitric oxide levels. In eNOS stimulated cells, 1400W brought down the levels to control + eNOS stimulation (Figure 15). This suggests that palmitate induced nitric oxide increase is through iNOS induction which marks the activation of inflammatory pathways.

3.3.5. Reactive oxygen species after free fatty acid treatment

Endothelial cells were incubated with sub-toxic levels (1µM, 5µM, 10µM, 25µM) of C2-ceramide for 24 hours (as described in 3.10) and DCFDA was added in order to determine the change in the levels of reactive oxygen species (ROS). In another set of experiments, high levels of C2-ceramide (150µM) were added to endothelial cells and ROS levels were monitored. Apocyanin, a mitochondrial ROS inhibitor was used at concentration 50µM to check the involvement of mitochondria in ROS generation.

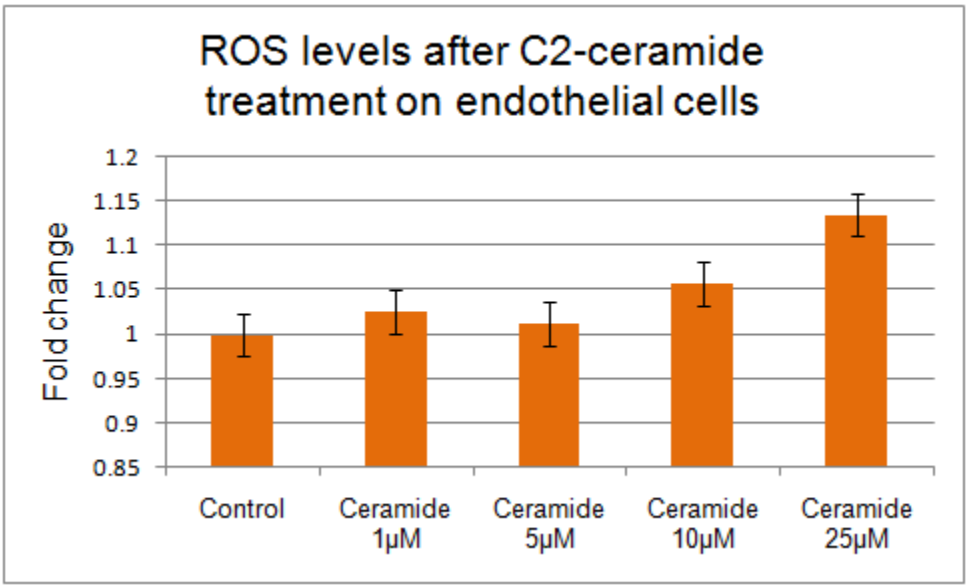

Figure 16: Change in the levels of reactive oxygen species (ROS) measured using DCFDA with flow cytometry after the treatment with different concentrations of C2-ceramide (1µM, 5µM, 10µM, 25µM). Data has been reported as Mean ± SD.

At the sub-toxic levels of C2-ceramide, no significant change in the levels of ROS was observed in endothelial cells (Figure 16). This suggests that free fatty acid derivates like ceramide might not be responsible for increasing oxidative stress *in vitro* at sub-toxic levels.

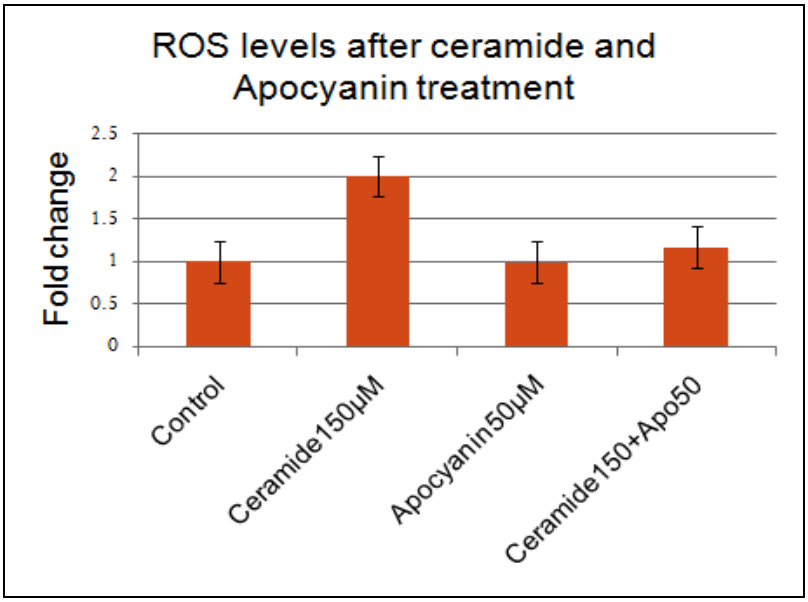

Figure 17: Change in the levels of reactive oxygen species (ROS) measured with the help of flow cytometry using DCFDA after the treatment high concentration of ceramide (150µM) and both control and ceramide treated sets were co-incubated with apocyanin (50µM). Data has been reported as Mean ± SD.

Treatment with high levels of ceramide (150µM) at which the viability of the cells were very low, increase in the levels ROS was observed. Treatment with mitochondrial ROS inhibitors like apocyanin decreased the levels of ROS suggesting the involvement of mitochondria in oxidative stress (Figure17).

3.3.6. Localization of palmitoyl-CoA and eNOS

To evaluate the modulation eNOS by free fatty acids if any, we treated the EA.hy926 human endothelial cells with 1µM palmitoyl-CoA tagged with FITC and stained with anti-eNOS and anti-rabbit TRITC antibody and nucleus stained with DAPI (described in 2.9).

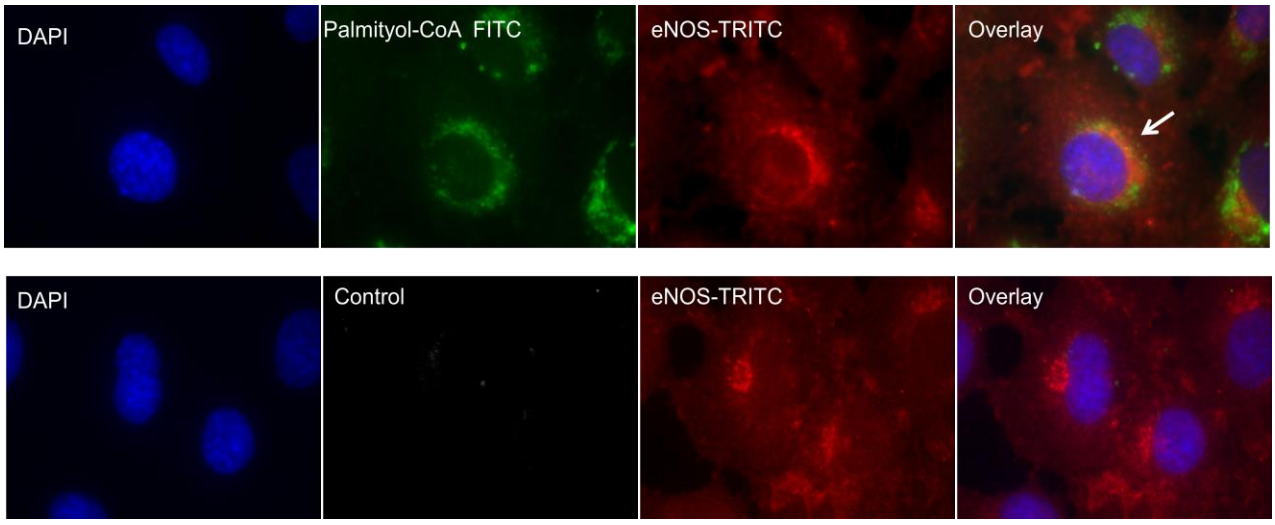

Figure 18: EA.hy926 human endothelial cells were treated with palmitoyl-CoA FITC conjugated, co-stained with anti-eNOS TRITC and DAPI. Arrow indicates the co-localisation of Palmitoyl CoA and eNOS.

Palmitoyl CoA and eNOS co-localized near the nucleus and suggests that there might be some interaction (Figure 18). Further experiments are required to check the direct effect of palmitate on normal functioning and phosphorylation of eNOS.

Chapter 5: Discussions

Type-2 diabetes mellitus (T2DM) is associated with much pathologies like hyper-tension, nephropathy etc. These pathologies are associated with high glucose and high free fatty acid in circulation. It is very difficult to understand molecular basis of diabetes occurrence, but there are fare evidences of what happens after the etiology of diabetes. All these etiologies are associated with high glucose and high FFA. Organs/tissues behave differently in response to these metabolic markers. Thus we tried to elucidate changes in cellular function in response to high glucose and high FFA.

We choose two cell types hepatocytes (liver epithelial cells) and endothelial cells in our experiments. Hepatocytes are good model system to understand metabolic syndrome and glycemic regulation as liver is one of the major organs involved in glucose metabolism and regulation. Evaluation of the effects of high glucose in the cultured hepatocytes is hence important. Although in order to understand the complete picture, these experiments need to be coupled with its effect of other organs/tissues like adipose, skeletal muscles etc.

In vitro H4IIE rat hepatocytes when treated with different concentrations of glucose retain viability maximum till 25mg/ml, but show a decline in viability at 50mg/ml. This concentration is different from the physiological concentration in body of humans. But *in vitro* hepatocytes can maintain their osmotic balance in high concentration of glucose. 25mg/ml and 50mg/ml of glucose was selected by us and then used in subsequent experiments to evaluate the effect of high glucose levels on inflammatory pathways. One such marker of inflammatory pathway modulator is expression of COX-2. Glucose was unable to induce any change in the expression of COX-2 in hepatocytes as observed in our experiments. This indicates that high glucose levels at sub-toxic concentration may not be the only factor in inducing inflammation or further still, COX-2 pathway might not be the one affected by higher levels of glucose.

In diabetic patients, it is observed that hyperglycemia is also associated with high levels of free fatty acid in circulation. So we evaluated the effect of free fatty acids on hepatocytes. Hepatocytes treated with sub-optimal levels of ceramides (5µM and 25µM)

did not induce COX-2 expression, but it increased the levels of nitric oxide released suggesting that free fatty acid derivatives like ceramide induce other inflammatory pathways responsible for patho-physiological conditions. There is strong literature evidence that free fatty acids induce inflammation, our results suggest that the activation is by COX-2 independent pathways and this in turn may be responsible for other patho physiological conditions. Thus in hepatocytes high glucose and high free fatty acids like ceramide does not induce COX-2 dependent inflammation. NOX pathway (induce nitric oxide pathway) is associated with higher ceramide synthesis as ceramide treatment on hepatocytes leads to nitric oxide production. Thus ceramides induce inflammation in hepatocytes by production nitric oxide in a COX-2 independent manner.

It is known that cardiovascular diseases are associated with the type-2 diabetes mellitus and inflammation is one of the major causes leading to such a patho-physiologic condition. The current hypothesis suggests that in endothelial system high levels of glucose and free fatty acids increase the free reactive oxygen species (ROS) levels that quench that eNOS dependent NO and thus adversely affect vasodilation and leads to endothelial dysfunction. We measured the ROS levels after free fatty acid treatment on endothelial system. Interestingly at sub-toxic levels of ceramide (FFAs) there was no significant change in the ROS levels. This disapproves with the current hypothesis that high ROS quenches eNOS dependent NO.

In order to check the effect of free fatty acids on endothelial cells we treated them with sub-toxic levels of ceramide (1µM, 5µM) and palmitate (100µM, 200µM). There was a significant increase in the levels of NO. Treatment with LNAME (non-specific NOS inhibitor) and 1400W (specific iNOS inhibitor) brought down the levels of nitric oxide suggesting the increase in the NO levels due to free fatty acid was iNOS dependent.

eNOS dependent NO is necessary for normal endothelial function but iNOS dependent NO can lead to endothelial dysfunction. This is majorly due to difference in the localization of NOS. As discussed earlier eNOS is localized in plasma membrane and golgi. It constitutively releases NO to the surrounding smooth muscles for vasodilation in Ca^{2+} independent manner. Whereas iNOS is localized in endoplasmic reticulum and

when induced due to high free fatty acids releases high levels of nitric oxide in Ca^{2+} dependent manner that can lead to dysfunction by nitrosylation of proteins and can change their function and conformation. Thus iNOS based NO release is deleterious and high levels of palmitate and ceramide induces iNOS based NO production that might have harmful affect on the normal function of endothelial cells.

As discussed earlier palmitate and other free fatty acids can modulate the expression of different enzymes in ceramide synthesis like SPT-2 (serine palmitoyl transferase-2), cerS etc. Palmitate conjugates to the serine sites present in these sites and thus modulates their functioning. eNOS also has three serine sites and thus we wanted to check if palmitate conjugates directly to the serine sites of eNOS and thus modulates its normal functioning. For this we performed palmitoyl uptake experiments in endothelial cells and co-stained them with eNOS antibody. Initial experiments suggest that palmitoyl-CoA and eNOS co-localize near nucleus. We need to perform more experiments on phosphorylation of eNOS under the effect of palmitate to conclusively talk about direct modulation and coupling of eNOS that might lead to dysfunction of eNOs and thus endothelial system.

In conclusion our data suggests that free fatty acids has significant role in contributing to systemic inflammation by inducing increase in the nitric oxide levels in hepatocytes and endothelial system and thus have deleterious effect. This increase in NO is through COX-2 independent manner. In endothelial cells, free fatty acid treatment did not increase ROS levels and thus it differs from current hypothesis of ROS quenching eNOS based NO. But free fatty acids increase the iNOS based NO in endothelial cells and might also be modulating eNOS coupling that decreases its function. But further experiments on phosphorylation of eNOS will give conclusive results.

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