Diabetes induced Oxidative Stress and the Development of Atherosclerosis: Role of Fatty Aldehyde Dehydrogenase

Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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Dedicated to
Lord Venkateshwara & my Parents

University of Hyderabad
(A Central University established in 1974 by act of parliament)
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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Diabetes induced Oxidative Stress and the Development of Atherosclerosis: Role of Fatty Aldehyde Dehydrogenase"** has been carried out by me under the supervision of Prof. P. Reddanna and Ranjan Chakrabarti Ph.D. This work has not been submitted for any degree or diploma of any other university earlier.

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This is to certify that **Mr. Juluri Suresh** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of the University. We recommend his thesis **"Diabetes induced Oxidative Stress and the Development of Atherosclerosis: Role of Fatty Aldehyde Dehydrogenase"** for submission for the degree of Doctor of Philosophy of this University.

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_____

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…..✍ ***Suresh***

ABBREVIATIONS

ALDH	**Aldehyde Dehydrogenase**
ABCA1	**ATP Binding Cassette Transporter Protein A1**
AGE	**Advanced Glycated End Product**
CAN	**Aceto Nitrile**
CBB	**Comassie Brilliant Blue**
CHCA	**Cinnnamic acid**
CM-H_2DCF-DA	**Carboxy-2'-7' dichlorofluroscein diacetate**
CML	**Carboxy Methyl lysine**
CVD	**Cardio Vascular Disorder**
Cu-Zn-SOD	**Copper Zinc Superoxide Dismutase**
eNOS	**Endothelial Nitric Oxide Synthase**
FFA	**Free Fatty Acid**
FAS	**Fatty Acid Synthase**
FALDH	**Fatty Aldehyde Dehydrogenase**
FADH	**Fatty Alcohol Dehydrogenase**
GSH	**Reduced Glutathione**
H_2O_2	**Hydrogen Peroxide**
HETE	**Hydroxyeicosatetraenoic Acid**
HVSMC	**Human Vascular Smooth Muscle Cells**
ICAM	**Intercellular Adhesion Molecule**
IKK	**Inhibitory Kappa Kinase**
IDDM	**Insulin Dependent Diabetes Mellitus**
IGF	**Insulin Growth Factor**
IRS	**Insulin Receptor Substrate**
INS	**Insulin**
LDL	**Low Density Lipoprotein**
Lox	**Lipoxygenase**
MCSF	**Macrophage Colony Stimulating Factor**

MMP9	**Matrix Metalloproteinase 9**
MCP-1	**Monocyte Chemoattractant protein 1**
NIDDM	**Non Insulin Dependent Diabetes Mellitus**
NFκB	**Nuclear Factor Kappa Beta**
PGG_2	**Prostaglandin G_2**
PGH_2	**Prostaglandin H_2**
PI3K	**Phosphatidylinositol-3-Kinase**
PEPCK	**Phosphophenol Pyruvate Carboxy Kinase**
ROS	**Reactive Oxygen Species**
RNS	**Reactive Nitrogen Species**
RAGE	**Receptor for Advanced Glycated End Product**
SMC	**Smooth Muscle Cells**
STZ	**Streptozotocin**
TNFα	**Tumor Necrosis Factor Alpha**
TFA	**Trifluro Acetic Acid**
Tro	**Troglitazone**
VEGF	**Vascular Endothelial Growth Factor**

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Changes in human lifestyle over the last century have resulted in a dramatic increase in the incidence of diabetes world wide. Diabetes mellitus is a complex syndrome involving severe insulin dysfunction in conjunction with gross abnormalities in glucose homeostasis and lipid metabolism. The term Diabetes Mellitus is derived from the Greek word which means 'sweet excessive urine'. Diabetes is a major global health problem which is now recognized by the WHO to be reaching epidemic proportions. It is the leading cause of death in most developed countries and is increasing rapidly in the developing countries like India, and China. The global figure of people with diabetes is set to rise from the current estimate of 150 million to 220 million in year 2010, and 300 million in the year 2025 due to pronounced changes in human lifestyle, behavior and environment. It is the cause of considerable morbidity and mortality mainly through cardiovascular disorders and to a minor extent through retinopathy (eye), nephropathy (kidney) and leg amputations (1998; Wild et al., 2004; Zimmet et al., 2001).

The disease is generally classified into two main forms. Type 1 or insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes. IDDM results from absolute insufficiency of insulin due to autoimmune-mediated destruction of insulin producing pancreatic β-cell islets, resulting in activation of gluconeogenesis, soaring blood glucose levels and gross loss of muscle and fat. People with type 1 diabetes must take exogenous insulin for survival to prevent the development of Ketoacidosis. It is currently affecting about 0.5% of the population in developed countries and increasing in incidence.

Type 2 or adult onset diabetes, or non insulin dependent diabetes mellitus (NIDDM) is typically a polygenic disease that results from a complex interplay between genetic predisposition and environmental factors such as diet, degree of physical activity and age. Type 2 diabetes is more complex in etiology and is characterized by relative insulin deficiency, reduced insulin action, and resistance to insulin mediated glucose transport in skeletal muscle and adipose tissue. To compensate, pancreatic β cells display increased secretion of insulin, resulting in hyperinsulinemia. Peripheral insulin resistance, in combination with impairment in the early phase of insulin secretion, results in hyperglycemia. In end stage type 2 diabetes leads to changes in insulin signaling, such as inability of insulin to inhibit hepatic gluconeogenesis and accompanied by deterioration of pancreatic β cell function and β cell exhaustion. People with type 2 diabetes are characterized with abnormalities like hyperglycemia, dyslipidemia (elevated triglycerides, low high density lipoproteins and increased low density lipoproteins), insulin resistance, and hypertension. People with type 2 diabetes are not dependent on exogenous insulin, but may require it for control of blood glucose levels if this is not achieved with diet alone or with oral hypoglycemic agents. The diabetes epidemic relates particularly to type 2 diabetes, and is taking place in both developed and developing nations (Ross et al., 2004).

Diabetic patients have an increased risk of development of various clinical complications due to microvascular or macrovascular diseases. The microvascular diseases in diabetes include nephropathy, retinopathy and neuropathy.

Macrovascular diseases include ischemic heart disease and atherosclerosis. The complications of macrovascular diseases are important cause of morbidity and mortality in type 2 diabetic patients. Among the abnormalities observed in the diabetic patients elevated glucose or hyperglycemia is now well established as a major factor contributing to the pathogenesis of atherosclerosis in diabetes. Hyperglycemia induces a large number of alterations at the cellular level of vascular tissue and increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that potentially accelerate the atherosclerotic process. Overproduction or insufficient removal of these free radicals results in vascular dysfunction, damage to cellular proteins, membrane lipids and nucleic acids. Four major biochemical mechanisms have been identified through which hyperglycemia induces free radicals.

1.1 Free Radical Generation by Hyperglycemia

1.1.1 Increased intracellular formation of Advanced Glycation End products (AGE)

AGE describes a heterogeneous group of proteins, lipids, and nucleic acids that are formed nonenzymatically through the reaction between extracellular proteins and glucose. Intracellular hyperglycemia is the primary initiating event in the formation of intra and extracellular AGEs. AGE formation starts with the reaction of the amino groups of proteins, particularly the side chains of lysine, arginine and

histidine, with reducing sugars, such as glucose, fructose, hexose-phosphates, trioses and triose-phosphates. The 'classic' or Hodge pathway begins with glucose condensation of amino groups to form a Schiff base aldimine adduct that undergoes rearrangement to a ketoamine Amadori product. This modification, termed 'non-enzymatic glycosylation', 'glycation' or 'Maillard reaction', leads to the formation of protein-bound Amadori products. Through subsequent rearrangements, dehydrations and oxidations, a heterogeneous group of fluorescent and brown products, the so-called 'advanced glycation end products' (AGEs) are formed. These compounds are more reactive than the parent sugars with respect to their ability to react with amino groups of proteins. The most important product of glycooxidation is carboxymethyllysine (CML) which is identified as a marker of local oxidative stress in tissues. The formation of AGE leads to chemical modification and cross linking of tissue proteins, lipids and DNA which affect their function, structure and lead to the pathogenesis of late diabetic complications (Nerlich and Schleicher, 1999). An amadori product forms H_2O_2 and other free radicals through two pathways. One pathway is the 1, 2 enolization pathway, which leads to 3-deoxyglucosone formation under anaerobic conditions. In the presence of a suitable electron acceptor, however, enolization would occur to H_2O_2 and glucosone. The other pathway is 2, 2 enolization pathway, which leads to 1- deoxyglucosone and the putative 1, 4-deoxyglcosone. Under oxidative conditions, however, the 2, 3-enediol is thought to generate H_2O_2 and carboxy methyllysine. 3-deoxyglucosones have been known to be major and highly reactive intermediates in the non-enzymatic glycosylation and a

potent cross- linker responsible for the polymerization of proteins to AGE. AGEs can be formed by non-oxidative and oxidative reaction pathways; the latter being significantly accelerated by transition metals, such as copper and iron. In this 'glycoxidation' reaction, protein-bound AGEs as well as soluble, highly reactive dicarbonyl products and oxygen free radicals are formed. Transition metals can also oxidise the monosaccharide directly in solution to form dicarbonyl products, which subsequently crosslink proteins through a process called 'autooxidative glycosylation'. AGE formation is irreversible and causes protease-resistant cross-linking of peptides and proteins (Brownlee, 1995; Elgawish et al., 1996; Wells-Knecht et al., 1995). The degree of AGE formation to a major extent is determined by the glucose concentration and the tissue redox potential. In diabetes, process of production of superoxide radicals by the transition metal catalyses autoxidation, followed by the dismutaion of superoxide to hydrogen peroxide. The generation of hydroxyl free radicals by Fenton reaction results in a site specific attack on proteins, leading to protein damage and damage to other cell components such as DNA (Brownlee et al., 1988; Lee et al., 1998; Mullarkey et al., 1990; Nishikawa et al., 2000; Schmidt et al., 1999; Yim et al., 1995).

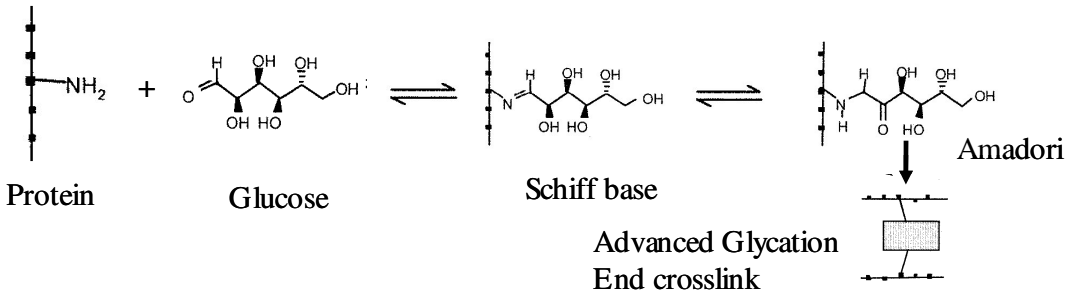


Figure 1. Chemical reaction leading to the formation of AGE (Adapted from Sladjana Dukic-Stefonovic: Biogerontology 2: 19-34, 2001).

The formation of AGE in diabetic patients leads to the acceleration of atherosclerosis by two mechanisms, non receptor mediated and receptor mediated.

1.1.1.1 Non receptor mediated mechanism:

Glycosylation of proteins and lipoproteins which are relevant to atherogenesis can interfere with their normal function by disrupting molecular conformation, alter enzymatic activity, reduce degrative capacity and interfere with the receptor recognition. One such lipoprotein is low density lipoprotein (LDL) whose normal function is altered due to glycosylation. The glycosylation process occurs on the apoprotein B mainly on a positively charged lysine residue within the putative LDL receptor binding domain which are essential for the specific recognition of LDL by the LDL receptor. Glycosylation of LDL results in impairment of LDL clearance from circulation as compared to the normal LDL through the LDL receptor. In contrast the glycated LDL is recognized by the

scavenger receptor expressed on the surface of macrophages and vascular smooth muscle cells leading to foam cell formation and increased cholesteryl esters (Bucala et al., 1994; Klein et al., 1995).

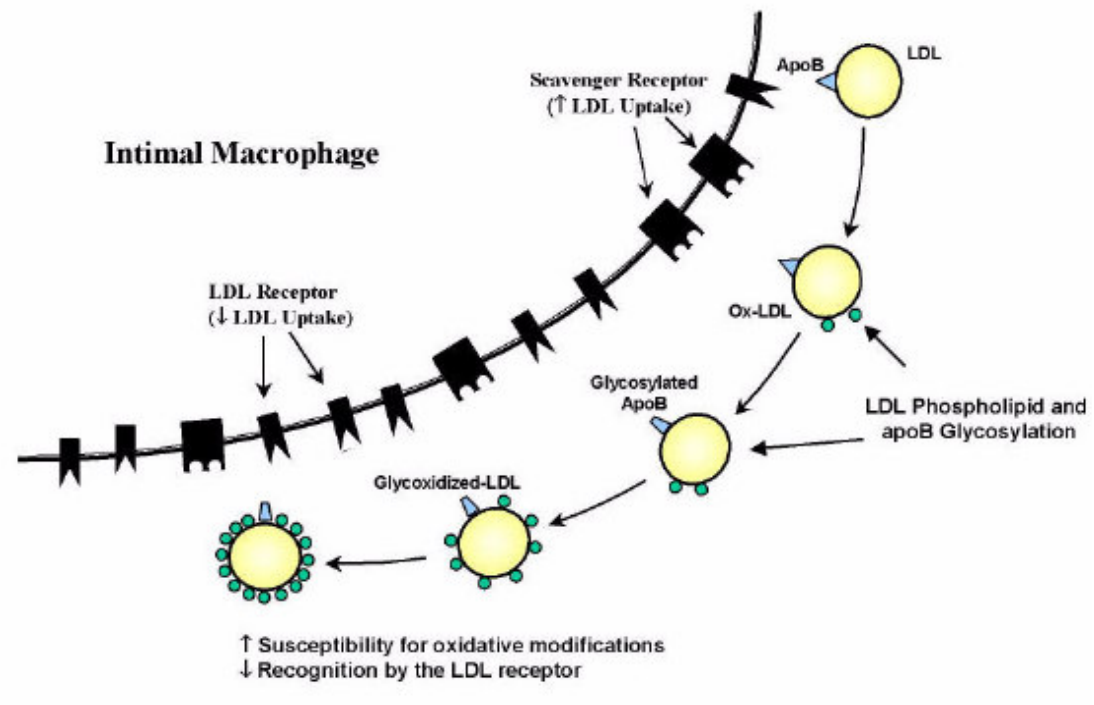


Figure 2. Non receptor mediated uptake of glycosylated LDL (Adapted from Doron Aronson et al., Cardiovascular Diabetology: 8 April, 2002)

1.1.1.2 Receptor mediated mechanisms:

The cellular interactions of AGEs are mediated through a specific receptor for AGE known as receptor for advanced glycosylation end product (RAGE). RAGE is a member of the immunoglobulin superfamily of receptors and is present on the

surface of cells relevant to the atherosclerotic process including monocyte derived macrophages, endothelial cells and smooth muscle cells. The macrophage AGE receptor system is closely tied to AGE turnover because in diabetic conditions there is a huge expression of RAGE in the cells where there is abundant AGE ligands (Ritthaler et al., 1995; Schmidt et al., 1999).

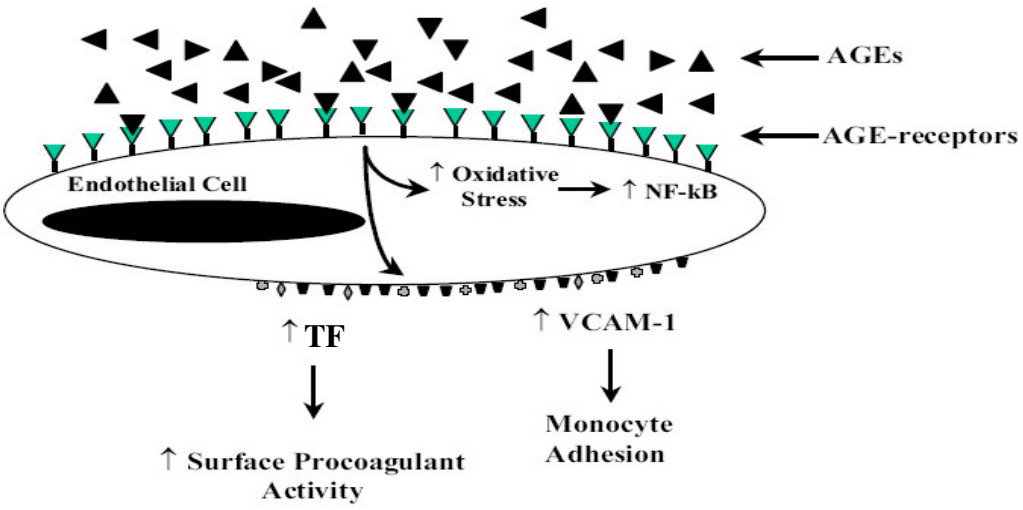


Figure 3. AGE interaction with the RAGE receptors. (Adapted from Doron Aronson et al., Cardiovascular Diabetology: 8 April, 2002)

Increased interaction of AGE with RAGE in macrophages results in the induction of oxidative stress and activation of the transcription factor NFκB as well as increased production of TNFα, interleukins and platelet derived growth factor. In addition interaction with RAGE on the endothelial cells results in increased permeability of

the monocytes in to the subendothelial space and differentiate into macrophages (Kirstein et al., 1990; Vlassara et al., 1988; Vlassara et al., 1992).

1.1.2 Increased polyol pathway flux:

Aldose reductase (Aldito: NAD $(P)^+$) is the first enzyme in the polyol pathway. It is a cytosolic, monomeric oxidoreductase that catalyses the NADPH-dependent reduction of a wide variety of carbonyl compounds, including glucose. Its crystal structure has a single domain folded into an eight stranded parallel α/β barrel motif, with the substrate binding site located in a cleft at the carboxy terminal end of the β barrel. In hyperglycemic condition, increased intracellular glucose results in its increased enzymatic conversion to the polyalcohol sorbitol, with concomitant decrease in NADPH. In the polyol pathway, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase, with NAD^+ reduced to NADH. Sorbitol does not diffuse easily across cell membrane and its accumulation intracellularly causes cell damage. Increased oxidation of sorbitol by NAD^+ increases the cytosolic NADH: NAD^+ ratio, thereby inhibiting activity of the enzyme glyceraldehyde 3 dehydrogenase (GADH), and increasing concentration of triose phosphate. Raised triose phosphate concentration increases the formation of AGE. Increased NAD^+/NADH ratio is linked to superoxide production via reduction of prostaglandin G_2 (PGG_2) to prostaglandin H_2 (PGH_2) by prostaglandin hydroperoxidase that uses NADH or NADPH as reducing co substrate. Reduction of glucose to sorbitol consumes NADPH. Because antioxidant enzymes like glutathione reductase require

NADPH, this process decreases reduced glutathione (GSH). GSH is one of the most important cellular antioxidants and is also involved in insulin metabolism. The net effect of this alteration in insulin processing is an increase in the oxidative stress (Baynes, 1991; Brownlee, 2001; Chappey et al., 1997).

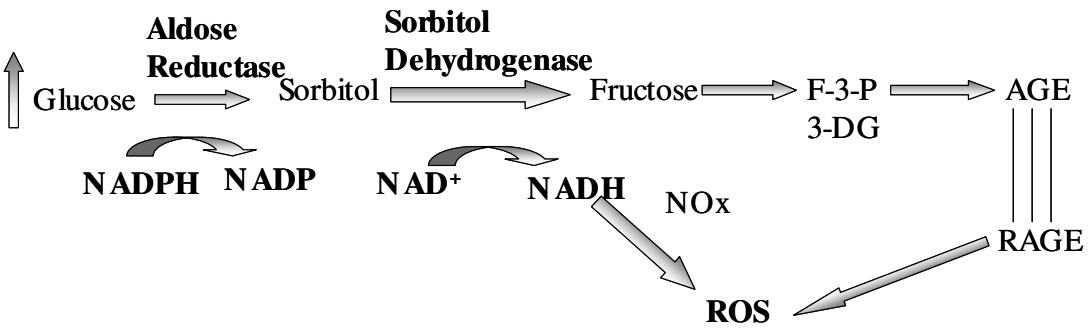


Figure 4. Polyol Pathway induced oxidative stress (Adapted from Stephens S.M. Chung et al., Journal of the American Society of Nephrology 14: S233-S236, 2003).

1.1.3 NADPH Oxidase:

NADPH oxidase is the major source of ROS production in the cardiovascular cells like macrophages and endothelial cells. NADPH oxidase is a membrane associated enzyme that catalyses the 1-electron reduction of oxygen using NADPH or NADH as an electron donor. The enzyme has five components p40phox (phox for Phagocyte Oxidase), p47phox, p67phox, p22phox and gp91phox. In the resting cell, three of these five components, p40phox, p47phox and p67phox exist in the cytosol forming a complex. The other two components p22phox and gp91phox are bound to

the membranes. When these two groups are separated as in the resting cell, the enzyme is inactive. During hyperglycemic conditions there is increase in the activity of the enzyme, leading to the production of ROS generation via PKC pathway (Hink et al., 2001; Kim et al., 2002).

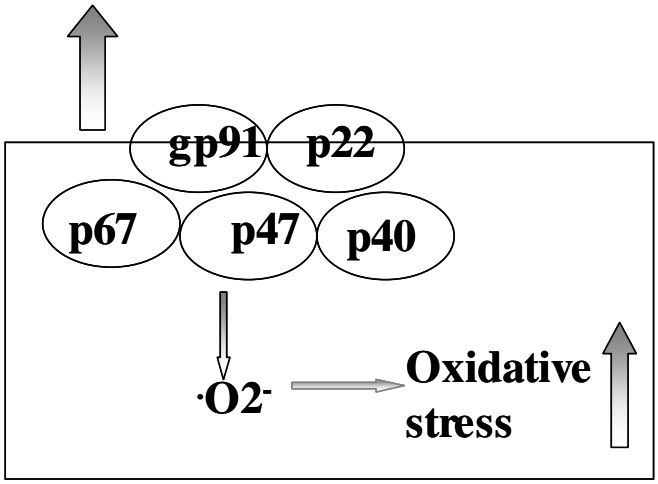


Figure 5. NADPH Oxidase System

1.2 Oxidative Stress

Oxygen is the primary oxidant in metabolic reactions designed to obtain energy from the oxidation of a variety of organic molecules. Oxidative stress results from the metabolic reactions that use oxygen, and is defined as excess formation and /or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). The definition implies that cells have intact pro-oxidant/antioxidant systems that continuously generate and detoxify

oxidants during normal aerobic metabolism. When additional oxidative events occur, the pro-oxidant systems outbalance the anti-oxidant potential producing oxidative damage to lipids, proteins, carbohydrates and nucleic acids leading to cell death in severe oxidative stress. ROS include free radicals such as superoxide anion ($^{\cdot}O_2^-$), hydroxyl ($^{\cdot}OH$), peroxyl ($^{\cdot}RO_2$), hydroperoxyl ($^{\cdot}HRO_2^-$) as well as non radical species such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Deby and Goutier, 1990). RNS include free radicals like nitric oxide ($^{\cdot}NO$) and nitrogen dioxide ($^{\cdot}NO_2^-$) as well as non radicals such as peroxynitrite ($ONOO^-$), nitrous oxide (HNO_2) and alkyl peroxynitrates (RONOO) (Evans et al., 2002). Of these reactive molecules only those reactive molecules which play important role in the diabetic cardiovascular complications are discussed below.

1.2.1 Superoxide anion radical ($O_2^{\cdot-}$):

Superoxide anion radicals are highly reactive free radicals generated during the normal oxidative phosphorylation in the mitochondria as a byproduct of normal cellular aerobic metabolism. In the mitochondrial electron transport system, donation of single electron to molecular oxygen results in the formation of the superoxide radical. In addition to this xanthine dehydrogenase/oxidase and NADPH oxidase are an important source of this free radical. The production of superoxide radicals at the membrane level (NADPH oxidase) is initiated in macrophages and contributes to their anti-bacterial action. The flavin cytosolic enzymes xanthine oxidase generates superoxide radicals from hypoxanthine and oxygen and is found to be one of the

causitive factors for the development of vascular pathogenesis. Superoxide reacts with nitric oxide generating highly cytotoxic secondary species known as peroxynitrite anion.

1.2.2 Hydrogen peroxide (H_2O_2):

Hydrogen peroxide is a non radical molecule (paired electron) generated mainly by enzymatic reactions. These enzymes are located in microsomes, peroxysomes and mitochondria. Even in normal conditions, the hydrogen peroxide production is relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. Normally superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\cdot-}$, thus contributing to the lowering of oxidative reactions. The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. H_2O_2 is able to diffuse easily through cellular membranes.

1.2.3 Hydroxyl radicals ($OH^{\cdot}$):

In the presence of a transition cation such as iron or copper, superoxide anion can give rise to the highly reactive hydroxyl radical species (OH.) by Haber - Weiss reaction. Iron catalyses the reaction and is considered the most prevalent reaction in biological systems and the source of various deleterious lipid peroxidation products. Hydroxyl radicals are considered to be a principal actor in the toxicity of partially

reduced oxygen species since it is very reactive with all kinds of biological macromolecules, producing products that cannot be regenerated by cell metabolism.

1.2.4 Nitric oxide (NO):

Nitric oxide is normally produced from L-arginine by endothelial nitric oxide synthase (eNOS) in the vasculature. Nitric oxide is produced by various types of cells and is well studied in vascular endothelium. Nitric oxide mediates endothelium dependent vasorelaxaiton by its action on guanylate cyclase in vascular smooth muscle cells initiating a cascade that leads to vasorelaxation. Nitric oxide also displays antiproliferative properties and inhibits platelet and leukocyte adhesion to vascular endothelium. While nitric oxide by itself is not reactive, even antioxidant under physiological concentration (upto 100nM), it reacts rapidly with superoxide produced in different pathological states and gives rise to extremely reactive peroxynitrite ($ONOO^-$) which mediates oxidation, nitrosation and nitration reactions. Nitric oxide is naturally formed in activated macrophages and endothelial cells and is considered as an active agent in several pathologies based on inflammation, organ reperfusion and also plays an important role in atherosclerosis.

1.3 Antioxidant System

Free radicals have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component in the surrounding. To protect the

cellular component from the harmful effects of the free radicals the cell has developed a defensive system known as antioxidant system. An antioxidant can be defined as "any substance that when present in low concentrations compared to that of an oxidisable substrate significantly delays or inhibits the oxidation of the substrate". The physiological role of antioxidants, as this definition suggests, is to prevent damage to cellular components arising as consequence of chemical reactions involving free radicals (Halliwell and Gutteridge, 1995). The antioxidant defense system can be divided into three main groups: antioxidant enzymes, chain breaking antioxidants, and transition metal binding proteins.

1.3.1 The Antioxidant Enzymes

1.3.1.1 Catalase:

Catalase is the first antioxidant enzyme to be characterized and catalyses the two stage conversion of hydrogen peroxide to water and oxygen.

$$\text{Catalase-Fe} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + H_2O_2 \longrightarrow \text{Catalase-Fe (III)} + 2H_2O + O_2$$

Catalase consists of four protein subunits each containing a heme group and a molecule of NADPH. Catalase is largely located within cells in peroxisomes, which also contain hydrogen peroxide. The amount of catalase in cytoplasm and other subcellular compartments remains unclear, because peroxisomes are easily ruptured during the manipulation of cells. The maximum activity is present in liver and

erythrocytes but some catalase is found in all tissues and cells (Aebi, 1984; Meilhac et al., 2000; Young and Woodside, 2001).

1.3.1.2 Glutathione peroxidase and glutathione reductase:

Glutathione peroxidase catalysis the oxidation of glutathione at the expense of hydroperoxide, which might be hydrogen peroxide or another species such as lipid hydroperoxide.

$$ROOH + 2GSH \longrightarrow GSSG + H_2O + ROH$$

Other peroxides including lipid hydroperoxides can also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. Several glutathione peroxidase enzymes are encoded by discrete genes. The highest concentrations are found in liver although glutathione peroxidase is widely distributed in almost all cells and tissues. The predominant subcellualr distribution is in the cytosol and mitochondria, suggesting that the glutathione peroxidase is the main scavenging enzyme of hydrogen peroxide in the subcellular compartments (Brigelius-Flohe, 1999; Nakane et al., 1998). The activity of the enzyme is dependent on the constant supply of reduced glutathione. The ratio of reduced to oxidized glutathione is usually very high as a result of the activity of the enzyme glutathione reductase.

$$GSSH + NADPH^{+} + H^{+} \longrightarrow 2GSH + NADP^{+}$$

The NADPH required for glutathione reductase is supplied by the pentose phosphate pathway. During the oxidative stress condition or in diabetic conditions there is high activity of the aldose reductase pathway which utilizes the NADPH, leading to the deficiency of reduced glutathione and hence impairs the action glutathione peroxidase. Glutathione reductase is a flavin nucleotide dependent enzyme and has a similar tissue distribution to glutathione peroxidase (Gibson et al., 1985; Young and Woodside, 2001).

1.3.1.3 Superoxide Dismutase:

The superoxide dismutase catalyses the dismutation of superoxide to hydrogen peroxides.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

The hydrogen peroxide is then removed by catalase or glutathione preoxidase. There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution.

(i). Copper Zinc Superoxide dismutase (CuZn- SOD): CuZN-SOD is found in the cytoplasm and organelles of virtually all mammalian cells. It has a molecular mass of

approximately 32000 KDa and has two protein subunits, each containing a catalytically active copper and zinc atom.

(ii). Manganese Superoxide dismutase (MnSOD): MnSOD is found in the mitochondria of almost all cells and has a molecular mass of 40000 KDa. It contains four protein subunits, each probably containing a single manganese atom. The amino acid sequence of MnSOD is entirely different to that of CuZn-SOD and is not inhibited by cyanide allowing MnSOD activity to be distinguished from that of CuZn-SOD in mixtures of the two enzymes.

(iii). Extracellular Superoxide dismutase (EC-SOD): It is a secretory copper and zinc containing SOD distinct from the CuZn-SOD. EC-SOD is synthesized by only few cell types, including fibroblast and endothelial cells, and is expressed on the cell surface where it is bound to the heparin sulphates. EC-SOD is the major SOD detectable in extracellular fluids and is released into the circulation from the surface of vascular endothelium following the injection of heparin. EC-SOD plays an important role in the protection of nitric oxide released from the endothelial cells from the superoxide radicals in the plasma (Karlsson et al., 1993; Marklund, 1982; McIntyre et al., 1999).

1.3.2 The Chain Breaking Antioxidants

Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts. In general the charge associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule, preventing further propagation of the chain reaction. Chain breaking antioxidants are divided into aqueous phase and lipid phase antioxidants.

1.3.2.1 Lipid Phase Chain Breaking Antioxidants:

Lipid phase chain breaking antioxidants scavenge free radicals in membranes, lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is vitamin E. Vitamin E occurs in eight different forms, which differ greatly in their degree of biological activity. The tocopherols (α, β, γ and δ) have a chromanol ring and a phtyl tail, and differ in the number and position of the methyl groups on the ring. They react more rapidly than polyunsaturated fatty acids with peroxyl radicals and hence act to break the chain reaction of lipid peroxidation. In addition to its antioxidant property, vitamin E plays a role in the stabilization of the membranes. α-tocopherol is the most potent antioxidant of the tocopherols and is also the most abundant in humans. It quickly reacts with the peoxyl radicals to form a relatively stable tocopheroxyl radical, with the excess charge associated with the chromonol ring. α-tocopherol is regenerated by

the reaction at the aqueous interface with the ascorbate or another aqueous phase chain breaking antioxidant such as reduced glutathione or urate (Kayden and Traber, 1993; May et al., 1998; Urano et al., 1992).

Another important antioxidant is the carotenoids, which are a group of lipid soluble antioxidant based around an isoprenoid carbon skeleton. The most important of these is the β- carotene. Carotenoids are efficient scavengers of singlet oxygen, but can also trap peroxyl radicals at low oxygen pressure with efficiency at least as great as that of α- tocopherol (Cooper et al., 1999; Fukuzawa et al., 1998).

1.3.2.2 Aqueous phase chain breaking antioxidants:

Aqueous phase chain breaking antioxidants directly scavenge the free radical present in the aqueous environment. Vitamin C (ascorbate) is the most important aqueous phase antioxidant. Ascorbate scavenges superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals and singlet oxygen. During its antioxidant action ascorbate undergoes a two electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate. Dehydroascorbate is relatively unstable and hydrolyses readily to diketogulonic acid, which is subsequently broken down to oxalic acid (Jialal et al., 1990). There are two mechanisms through which dehydroascorbate are reduced back to ascorbate; one is mediated by the selenoenzyme thioredoxin reductase (May et al., 1998) and the other is non enzyme mediated reaction that uses reduced glutathione (May et al., 1996).

Other than antioxidant property ascorbate acts as an essential cofactor for several enzymes catalyzing hydroxylation reactions.

Other important antioxidants in this class apart from ascorbate are Uric acid and reduced glutathione. Uric acid efficiently scavenges radicals, being converted in the process to allantion. Urate is particularly important in providing protection against certain oxidizing agents such as ozone (Grootveld and Halliwell, 1987).

Reduced glutathione (GSH) is a major source of thiol groups in the intracellular compartment but is of little importance in the extracellular space. GSH might function directly as an antioxidant, scavenging a variety of radical species as well as acting as an essential cofactor for glutathione peroxidases (Young and Woodside, 2001).

1.3.3 The Transition Metal Binding Proteins:

The transition metal binding proteins (Ferritin, transferrin, lactoferrin and ceruloplasmin) act as crucial component of the antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. The main copper binding protein, ceruloplasmin also function as an antioxidant enzyme that can catalyse the oxidation of divalent iron

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

Fe^{2+} is the form of iron that drives the fenton reaction and the rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant (Atanasiu et al., 1998; Young and Woodside, 2001).

1.4 Oxidative Stress and Inflammation:

Hyperglycemia induced oxidative stress followed by inactivation of the antioxidant systems, along with soluble AGEs and products of lipid peroxidation serve as key activators of stress sensitive intracellular signaling pathway in the macrophages. Activation of stress signaling pathway leads to the induction of inflammatory gene products that cause cellular damage and are ultimately responsible for the late complications of diabetes like atherosclerosis (Evans et al., 2002; Lin et al., 2005). One major intracellular target of hyperglycemia and oxidative stress is the redox sensitive transcription factor nuclear factor-κB (NF-κB). NF-κB can be activated by a wide array of exogenous and endogenous stimuli including hyperglycemia, ROS, AGE binding to RAGE, and elevated FFA. NF-κB plays a critical role in mediating immune and inflammatory responses and apoptosis. The aberrant regulation of NF-κB is associated with a number of chronic diseases including diabetes and atherosclerosis.

NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of p50 and p65 subunits complexed with an inhibitor protein subunit, IκB. After stimulation, a serine kinase cascade is activated leading to the phophorylation of IκB

and leading to substrate ubiquitination and subsequent degradation, freeing the NF-κB heterodimer to translocate to the nucleus. Once migrated to the nucleus NF-κB regulates the expression of large number of genes, including growth factors like

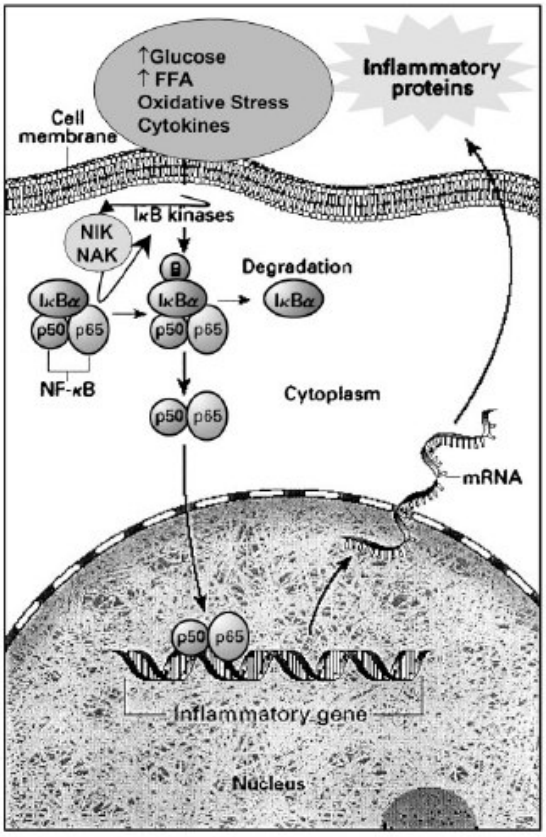


Figure 6. NF-κB activation by Hyperglycemia (Adapted from Joseph L. Evans et. al., Endocrine reviews 23(5): 599-622, 2002).

vascular endothelial growth factor (VEGF), proinflammtory cytokines (TNFα, IL-6, and IL-1β), RAGE, adhesion molecules (VCAM-1, ICAM-1) which are involved in the development of atherosclerosis (Baldwin, 2001; Bowie and O'Neill, 2000a; Collins and Cybulsky, 2001; Valen et al., 2001). An increase in the presence of the

NFκB regulated inflammatory mediators such as cytokines, inducible nitric oxide synthase and adhesion molecules in the atherosclerotic lesions has been reported (Barath et al., 1990; Buttery et al., 1996; Frostegard et al., 1999).

The enzyme that phosphorylates IκB is IκB kinase (IKK), a heterodimeric complex consisting of two catalytic subunits, IKKα and IKKβ and a regulatory subunit, IKKγ. IKK is activated after serine phosphorylation catalyzed by upstream serine kinase, including NF-κB inducing kinase (NIK) and NF-κB activating kinase (NAK). Although both IKKα and IKKβ subunits are subject to serine phosphorylation, inhibition of phosphorylation of serine sites in IKKβ prevents the activation of total IKK activity (DiDonato et al., 1997; Ling et al., 1998; Mercurio et al., 1997; Tojima et al., 2000). Phosphorylation of the serine residues on IKKβ is directly inhibited by various antioxidants like vitamin E, C and Lipoic acid. Even drugs like aspirin, salicylate and thiazolidinediones show the anti-inflammatory effect by inhibiting the phosphorylation of the IKKβ (Bowie and O'Neill, 2000b; Hamuro et al., 2002; Kim et al., 2007; Lehmann et al., 1995; Pieper et al., 2002; Saeed et al., 2003; Saltiel and Olefsky, 1996; Schubert et al., 2002; Yin et al., 1998).

Nuclear factor kappa-B plays an important role in the development of atherosclerosis by transducing the pathogenic stimulation originating from oxidative stress (ROS) to the expression of genes that promote recruitment and activation of inflammatory cells in the artery. Activation of NFκB after stimulation with reactive oxygen species have been reported in macrophages, vascular smooth muscle cells and endothelial cells in human atherosclerotic plaque of diabetic patients but not in

healthy people by various researchers (Bourcier et al., 1997; Brand et al., 1996; Schreyer et al., 1996).

1.4.1 Atherosclerosis:

Atherosclerosis, formerly considered a bland lipid storage disease, actually involves an ongoing inflammatory response. Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of the diseases from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis. The new finding suggests that inflammation is the key link between diabetes and atherosclerosis (Libby et al., 2002; Ross, 1999).

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. The presence of fatty deposits, called plaques, leads to loss of arterial elasticity with narrowing of the artery. The constriction to smooth blood flow deprives vital organs of their blood supply. Clots may lodge in arteries supplying the heart, causing myocardial infraction (heart attack), or the brain, causing stroke (Lusis, 2000). The major targets of atherosclerosis are the aorta of the coronary and cerebral arteries. The three basic processes leading to the formation of atherosclerotic lesion are: (i) invasion of the artery wall by monocytes and T-lymphocytes; (ii) smooth muscle cell (SMC) phenotypic modulation, proliferation and synthesis of extracellular matrix; and (iii)

intracellular lipoprotein uptake in macrophage and SMC, and lipid accumulation (Campbell and Campbell, 1997).

1.4.1.1 Lesion Initiation:

A large artery consists of three morphologically distinct layers. The intima, the innermost layer, is bounded by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibers, the internal elastic lamina, on the pheripheral side. The normal intima is a very thin region and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The media, the middle layer consists of smooth muscle cells (SMC). The adventia, the outer layer, consists of connective tissue with interspersed fibroblasts and SMCs. The initiation of atherosclerosis occurs due to damage to the endothelial lining of the artery which forms a tight intracellular juncional complex and functions as a selective permeable barrier between blood and tissue. Damage to the endothelial cells might be because of excess production of reactive oxygen species (oxidative stress) or fluid shear stress leading to inactivation of important function of endothelial cells like regulating thrombosis, inflammation, vascular tone and vascular remodeling (Gimbrone, 1999; Ross, 1993). The result of endothelial cell injury is increased permeability of LDL, increased expression of RAGE receptors, increased adhesion of monocytes, leukocytes to the cell adhesion and migration and accumulation in the subendothelial space. In the sub endothelial space monocytes transform into macrophages due to the

effect of macrophage colony stimulating factor (MCSF) secreted by the endothelial cells.

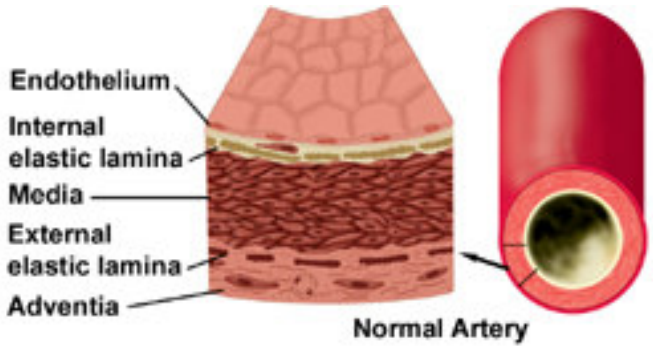


Figure 7. Cross section of normal artery

The differentiated macrophages secrete (a) Monocyte chemoattractant protein (MCP-1) which is important in recruiting monocytes from the circulation into the subendothelial space, (b) ROS production due to increased activity of NADPH oxidase, inducible nitric oxide synthase, and lipoxygenase. The ROS causes LDL oxidation and reacts with nitric oxide leading to the formation of highly reactive peroxy nitrite radicals and inhibits the nitric oxide synthesis from the endothelial cells (Boren et al., 1998; Cyrus et al., 1999; Dong et al., 1998; Goldstein et al., 1979; Gu et al., 1998; Mehrabian et al., 2002; Ni et al., 2001; Shi et al., 2002; Skalen et al., 2002; Steinberg and Witztum, 2002).

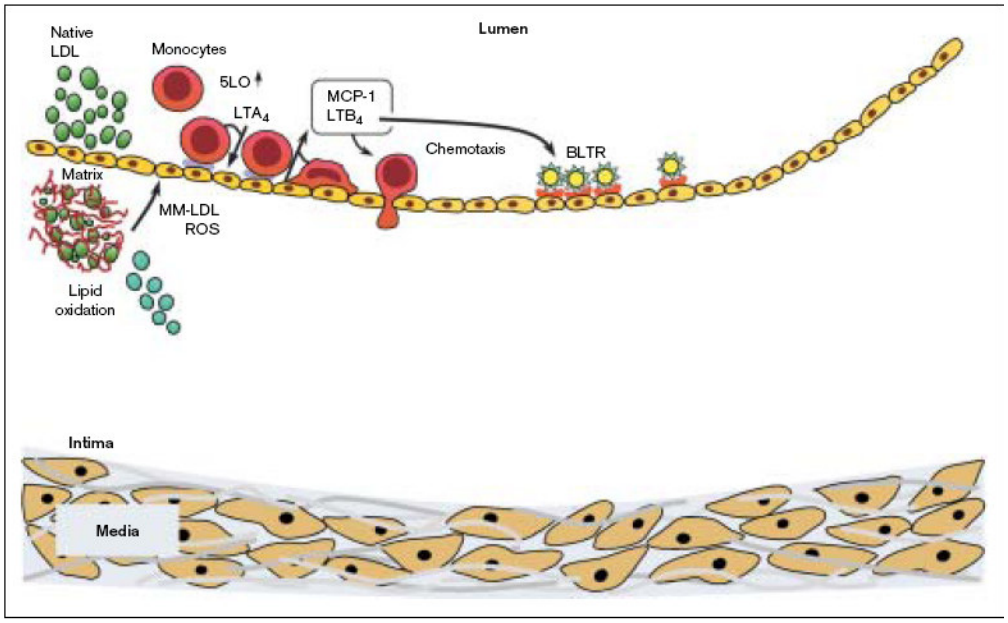


Figure 8. Initiation of atherosclerotic lesion (Adapted from Margerete Mehrabian et al., Current Opinion in Lipidology, 14, 447-457, 2003).

1.4.1.2 Foam cell Formation:

Macrophages express several scavenger receptors that are capable of taking up oxidized LDL, including scavenger receptor A, scavenger receptor B1, CD36 and CD68.The expression of the scavenger receptor is regulated by the MCSF secreted by the endothelial cells and by cytokines such as TNFα and interferon- γ (Smith et al., 1995; Tontonoz et al., 1998). Scavenger receptors do not take up native LDL but take up oxidized LDL more readily and lead to the formation of foam cells. Oxidized phospholipids are found to be important determinants for the recognition oxLDL by scavenger receptors (Febbraio et al., 2001; Linton and Fazio, 2001).

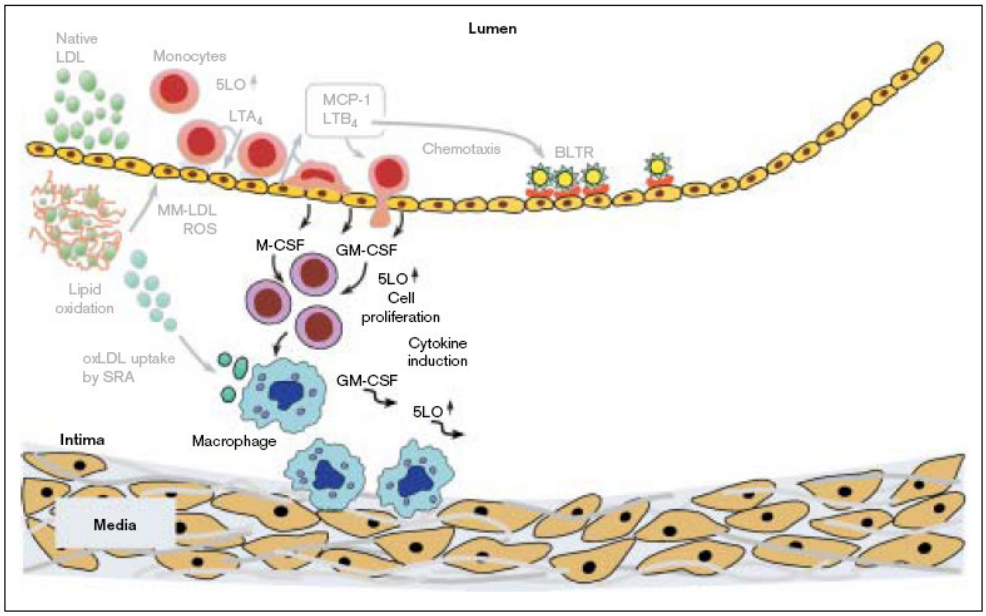


Figure 9. Macrophage accumulation and foam cell formation in the lesion (Adapted from Margerete Mehrabian et al., Current Opinion in Lipidology, 14, 447-457, 2003).

1.4.1.3 Fibrous Plaque and Thrombosis:

Fibrous plaque is characterized by a growing mass of extracellular lipid, mostly cholesterol and its ester, and by the accumulation of SMCs and SMC derived extracellular matrix. Cytokines and growth factors secreted by macrophages and T cells are important for SMC migration and proliferation and extracellular matrix production. The intimal SMCs secrete extracellular matrix and give rise to fibrous cap (Lusis, 2000). Development of thrombosis or rupturing of the plaque depends on composition and vulnerability rather than the severity of stenosis.

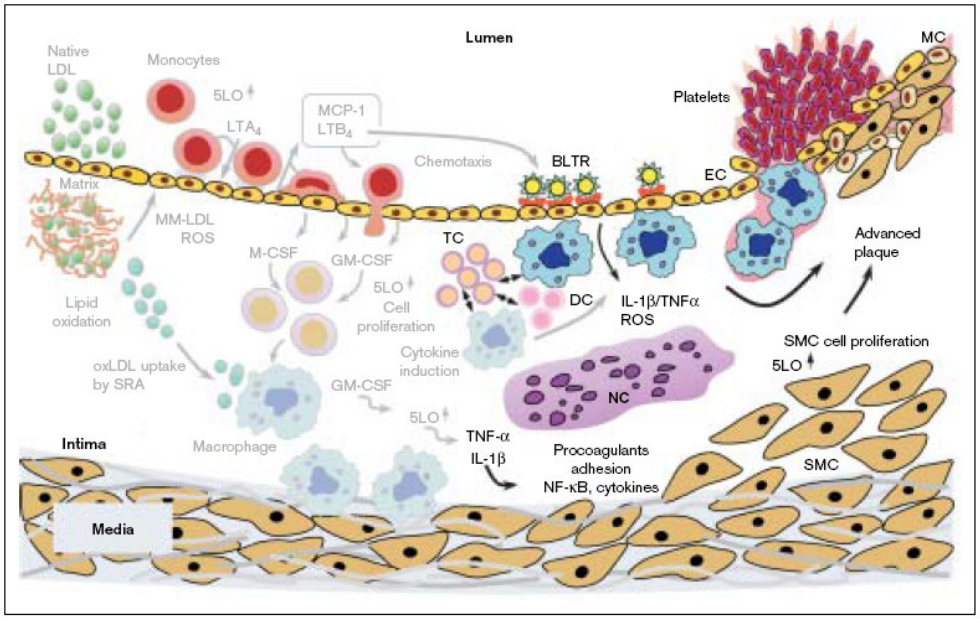


Figure 10. Progression to advanced atherosclerotic lesion (Adapted from Margerete Mehrabian et al., Current Opinion in Lipidology, 14, 447-457, 2003).

Products of inflammatory cells like macrophages and T cells are responsible for the stability of the plaque. Macrophages secrete cytokines like INFγ which inhibit the SMCs from the production of matrix and matrix degrading proteases like matrix metalloproteinase- 9 (MMP9) which degrade the matrix. Rupture frequently occurs at the lesion edges, which are rich in foam cells, suggesting that factors contributing to inflammation also influence thrombosis. Thrombogenicity of the lesion also occurs due to increased production of tissue factor by endothelial cells and macrophages. Production of the tissue factor is enhanced by oxLDL and the ligation

of CD40 on endothelial cells to CD40L on macrophages and T-cells (Lusis, 2000; Schonbeck et al., 2000; Watson et al., 1994).

Oxidative stress induced inflammation is not only associated with development of atherosclerosis, but also involved in inhibiting the insulin signaling pathway thereby causing insulin resistance. Insulin affects cells by binding to its receptor on the surface of the insulin responsive cells such as muscle, adipocytes, macrophages, endothelium and vascular smooth muscle cells.

1.5 Insulin Signaling:

The insulin receptor is a transmembrane tyrosine kinase receptor able to form homo or heterodimers with the IGF receptor as disulfide linked $\alpha 2\beta 2$ tetramer proteins. Insulin binds with high affinity to the subunit of the insulin receptor, leading to subsequent autophosphorylation of the β subunits on three intracellular tyrosine residues and followed by phosphorylation of several insulin receptor substrates (IRS) family. The insulin receptor phosphorylates at least nine intracellular signaling molecules including four intracellular insulin receptor substrates (IRS proteins IRS1,-2, -3, and 4). Phosphorylated tyrosine residue on each of these substrate proteins enables docking of a distinct subset of downstream signaling molecules containing Src homology-2 domains. Docking proteins include the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K) adapter protein Grb2, Nck and Shc, protein tyrosine and tyrosine kinase.

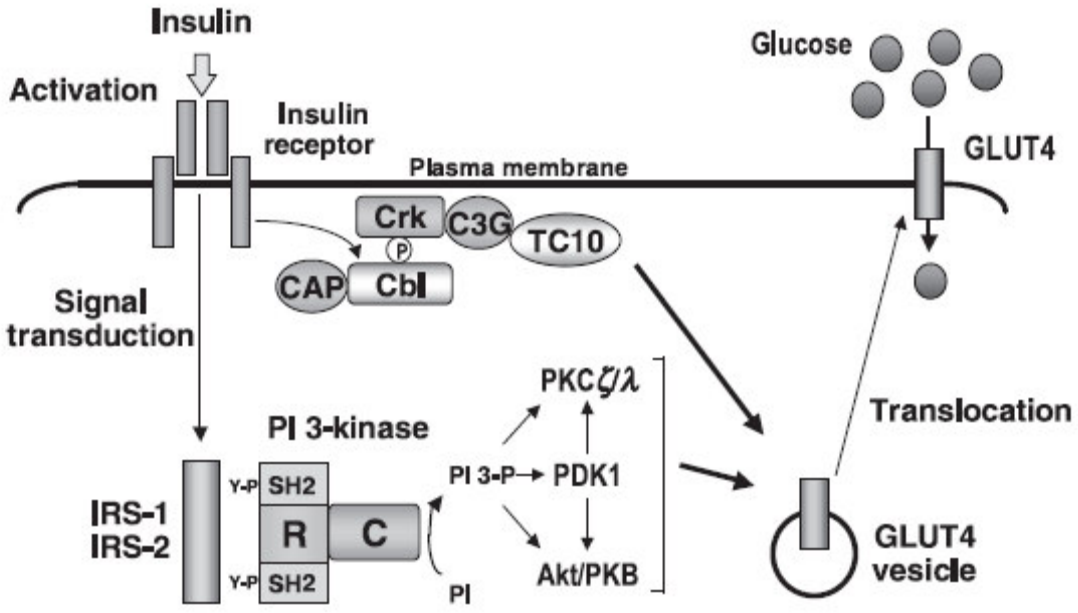


Figure 11. Insulin Signaling Pathway (Adapted from Giorgino.F et al., Acta Physiol Scand, 183, 13-30, 2005).

These proteins thus initiate the downstream signaling events through the PI3K which is a key component of the intracellular insulin signaling machinery (Federici et al., 1997; Pessin and Saltiel, 2000; Saltiel and Kahn, 2001; Skolnik et al., 1993; Sun et al., 1996; White, 1997).

The inhibition of signaling downstream of the insulin receptor is the primary mechanism through which inflammatory mediators lead to insulin resistance. TNFα cytokine stimulates the inhibitory phosphrylation of serine residues of IRS-1. This phosphorylation reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability of IRS-1 to associate with the insulin receptor and thereby

inhibits downstream signaling and insulin action (Aguirre et al., 2000; Aguirre et al., 2002; Hotamisligil et al., 1996; Paz et al., 1997). Activation of IKKβ which is the key regulator of the NFκB activation also leads to insulin resistance through two pathways. First, it can directly phosphorylate IRS-1 on serine residues. Second , it can phosphorylate inhibitor of NFκB (IκB), thus activating NFκB, a transcription factor that stimulates the production of inflammatory cytokines like TNFα and IL6 which causes insulin resistance (Gao et al., 2002; Yin et al., 1998).

PI3K modulates the expression of number of genes like PEPCK, Glucose 6 phosphate, Hexokinase, glucokinase, FAS, and Glut-4 which are involved in lipid and glucose homeostasis. (Dickens et al., 1998; Kang et al., 2003; Wang and Sul, 1998).

1.6 Fatty Aldehyde Dehydrogenase (FALDH)

FALDH is one of the genes regulated by PI3K and catalyses the irreversible oxidation of a broad class of aldehydes to their corresponding carboxylic acids, products that tend to be much less toxic than the parent aldehydes (Vasiliou et al., 2004; Vasiliou et al., 2000). Lipid peroxidation produces a large number of aldehydes, alcohols and ketones as byproducts which are cytotoxic to the cells and have to be detoxified immediately. Aldehydes are organic compounds containing a terminal carbonyl functional group with at least one proton attached.

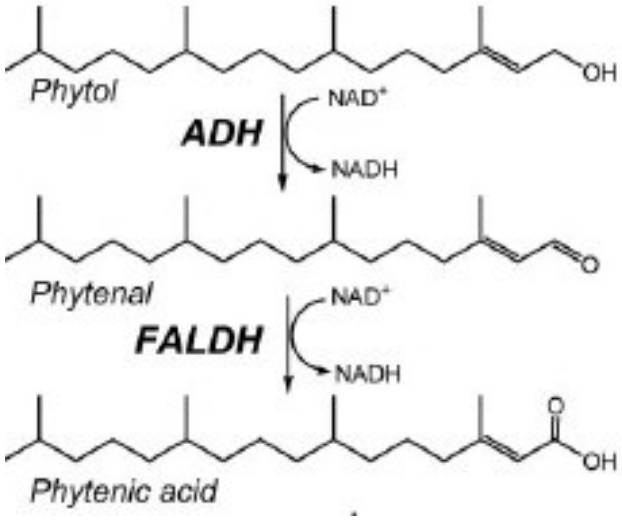


Figure 12. Oxidation of Fatty aldehydes to fatty acid by Fatty Aldehdye Dehydrogenase (FALDH)

The presence of α-hydrogen increases the reactivity of the carbonyl group such that the carbon develops a partial positive charge leading to an increase in electron density over the oxygen atom. Because of such charge delocalization the terminal carbonyl functional group can interact with amine groups (e.g. terminal groups of lysine and arginine) forming Schiff base. The aldehydes carbonyl also forms thioester bonds with nucleophilic cysteine residues. The presence of α, β unsaturation in the aldehydes increases the reactivity with electron rich centers of proteins and non proteins forming Schiff base. This dual reactivity is a central feature of the biological activity of aldehydes and is the underlying mechanism of their toxicity (Conklin et al., 2007). Accumulation of aldehydes leads to stimulation of stress signaling pathway, derive macrophage uptake of oxidized LDL in

subintimal space, increase production of ROS and cytokines in macrophages and other cells involved in the atherosclerosis progression (Akhand et al., 2001; Chang et al., 2005; Fan et al., 2003; Lusis, 2000).

Aldehyde Dehydrogenase (ALDH) are a group of NAD $(P)^+$ dependent enzymes involved in the metabolism of a wide variety of aliphatic and aromatic aldehydes. ALDH proteins are conveniently classified into families and subfamilies based on the percentage of amino acid identity. The human genome contains 17ALDH genes arranged into 10 families and 13 subfamilies. Many allelic variants exist within these gene families, resulting in pharmacogenetic heterogeneity between individuals which, in most cases, results in a distinct phenotype (Vasiliou et al., 2004). Mammalian tissues express several ALDHs that catalyse the irreversible oxidation of a broad class of aldehydes to their corresponding carboxylic acids, products that tend to be much less toxic than the parent aldehydes.

1.6.1 Classification of Aldehyde Dehydrogenase:

Mammalian ALDHs are classified according to their subcellular localization, biophysical and kinetic properties, and primary sequence similarity.

(i) The Class 1 ALDH1s include both constitutively and inducible isoforms encoded by at least 5 separate genes. These genes are currently used in screens for potentially useful anti cancer agents due to their decreased expression in certain types of tumors (Conklin et al., 2007).

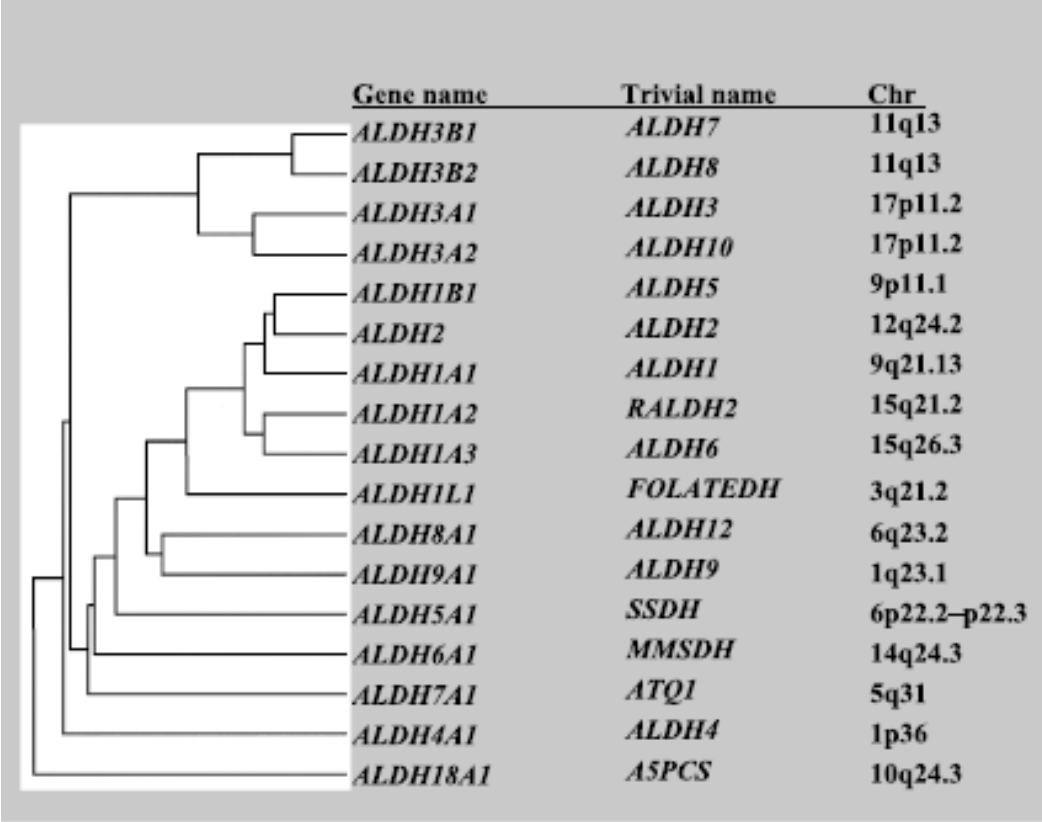


Figure 13. Clustering dendrogram and chromosome localization of human aldehyde dehydrogenase genes (Adapted from Vasiliou Vasilis et. al., Drug Metabolism Reviews 36, 279-299, 2004).

(ii) The Class 2 ALDH2 gene encodes a mitochondrial enzyme that displays the highest affinity for acetaldehyde as substrate like any other member of the super family. This enzyme is expressed in high concentration in the liver and in tissues

which depend on mitochondria for energy metabolism (e.g. heart) (Peng et al., 1999; Vasiliou et al., 2000).

(iii) The class 3 ALDH (ALDH3) includes tumor specific and inducible cytoplasmic ALDH3A1 along with constitutive microsomal ALDH3A2. Both are expressed in virtually all tissues and are involved in the metabolism of fatty aldehydes. The class 3 ALDHs is widely distributed in extra hepatic tissues, including cardiovascular tissues and prefers aromatic aldehydes and medium chain aliphatic aldehydes as substrates. The ALDH3 are involved in the metabolism of aldehydes formed during lipid peroxidation. The ALDH3A2 gene encodes the fatty aldehyde dehydrogenase (FALDH; also known as microsomal ALDH) (Dunn et al., 1988; Liu et al., 1997; Vasiliou et al., 2000). Fatty aldehyde dehydrogenase is a microsomal enzyme that catalyses the oxidation of medium and long chain aliphatic aldehydes derived from metabolism of fatty alcohol, phytanic acid, ether glycerolipids and leukoteriene B4. The FALDH gene in man and mouse consists of 11 exons, 10 introns and is closely linked to the gene for ALDH3. In both species, alternative splicing results in the formation of a second minor protein, FALDHv, that has a unique carboxy terminal end. The functional significance of this alternate protein is not known. FALDH is a component of the fatty alcohol: NAD^+ oxidoreductase complex (FAO). FAO is a complex enzyme which consists of two separate proteins that sequentially catalyze the oxidation of fatty alcohol to fatty aldehydes and fatty acid; reactions are catalysed by fatty alcohol dehydrogenase (FADH) and fatty aldehyde dehydrogenase

respectively. FALDH has a molecular weight of 54 KDa and is synthesized on free polysomes and then post translationally inserted into the endoplasmic reticulum. The enzyme normally resides on the outside of the endoplasmic membrane. The active site of the enzyme is usually exposed towards the cytoplasm side.

Mutations in the human ALDH3A2 gene lead to reduced activity of FALDH and is the molecular basis underlying Sjogren-Larsson syndrome (SLS) an autosomal recessive disorder characterized by congenital icthyosis, mental retardation, spasticity, ocular abnormalities and pruritus (Chang and Yoshida, 1997; Kelson et al., 1997; Mitchell and Petersen, 1989; Rizzo and Craft, 1991; Rizzo et al., 2001; Rogers et al., 1997; Willemsen et al., 2001; Willemsen et al., 1999). Demozay *et. al* (Demozay et al., 2004) has reported that ALDH3A2 gene is under the control of insulin and is upregulated in the liver and adipose tissue after insulin injection. FALDH mRNA levels are downregulated in the liver and adipose tissue of type 1 diabetic model (streptozotocin treated rats) and type 2 diabetic model (*db/db* mice), suggesting that ALDH3A2 deregulation occurs both in hyperinsulinemic insulin resistant state and in hypoinsulinemic type 1 diabetic model. However, the underlying mechanism involved in the deregulation of FALDH in the diabetic condition is not yet known.

1.7 The Scope of the Present Study:

Cardiovascular complications characterized by endothelial dysfunction and accelerated atherosclerosis are the leading cause of mortality and morbidity associated with diabetes. Abnormalities in diabetes like hyperglycemia induces a large number of alterations at the cellular level and leads to the generation of highly reactive free radicals as reactive oxygen species (ROS) and reactive nitrogen species (RNS) through various mechanisms, which ultimately causes oxidative stress. Increased oxidative stress due to hyperglycemia causes alterations /disturbances in the cellular redox state and leads to the activation of stress sensitive intracellular signaling pathway in the macrophages. Activation of stress signaling pathway induces the expression of large number of genes, viz proinflammatory cytokines and others that are involved in the development of atherosclerosis. Elevated levels of TNFα causes insulin resistance and disrupts insulin induced signaling events through phosphatidylinositol 3- kinase (PI3 Kinase) pathway. Insulin acts via PI3 kinase by modulating the expression of a number of genes involved in lipid and glucose homeostatisis.

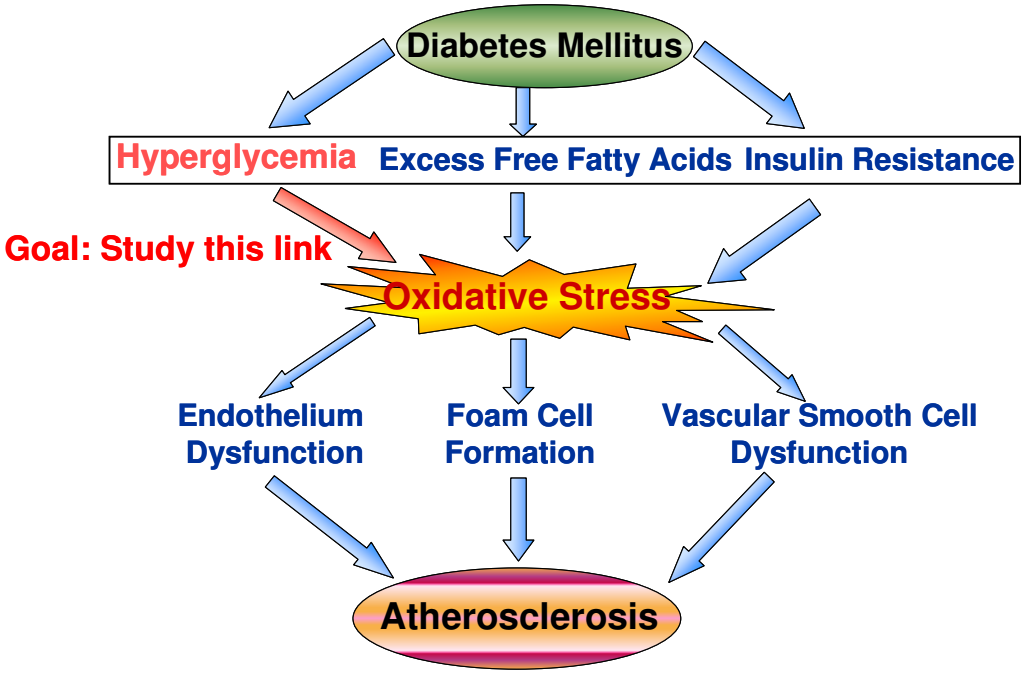
Fatty Aldehyde Dehydrogenase (FALDH) is one of the genes regulated by PI3K and catalyses the irreversible oxidation of a broad class of aldehydes to their corresponding carboxylic acids, products that tend to be much less toxic than the parent aldehydes. FALDH is an important detoxifying enzyme of both exogenous and endogenous aldehydes such as those derived from lipid peroxidation, glucose oxidation and oxidative stress in diabetes. Aldehydes accumulation leads to the

stimulation of stress signaling pathways, increases production of ROS and cytokine in macrophages and endothelial cells which are involved in the atherosclerosis progression. FALDH is regulated by insulin and is downregulated in diabetes; however the underlying mechanism responsible for the deregulation of FALDH is not yet known.

Since FALDH is an important detoxifying enzyme and protects the cells from the cytotoxic effects of aldehydes, the present study hypothesizes that increased abundance of aldehydes in the cardiovascular tissue of diabetic patients might be due to the loss of FALDH activity. The loss of FALDH activity might be due to hyperglycemia induced oxidative stress. Since aldehyde accumulation causes cardiovascular complications mainly through the modulation of macrophage functioning, in the present study experiments were carried out in the macrophages**.** Hyperglycemia induced oxidative stress and the regulation of FALDH gene was studied using RAW 264.7 mouse macrophages. This study reports for the first time the presence of FALDH gene in macrophages and its downregulation in response to hyperglycemia induced oxidative stress. The downregulation of FALDH could be reversed by insulin, ascorbic acid (antioxidant) and troglitazone (antidiabetic drug) confirming the role of ROS in FALDH modulation. Further *in vivo* FALDH modulation was studied in the aorta of diabetic animal models and significant reduction of FALDH expression in both type 1 and type 2 diabetic mice was observed. These results confirm that the increased accumulation of aldehydes in the aorta is due to downregulation of FALDH detoxifying system. The data obtained at

the gene level was further confirmed at the protein level by proteomic studies. The 2D gel analysis and modulation at the protein level is being reported for the first time further confirming that the downregulation of FALDH is mediated through hyperglycemia induced oxidative stress. Thus the proposed mechanism for FALDH downregulation involves hyperglycemia induced ROS and the production of TNFα by macrophages.

Hypothesis of Present Work:



2.1 Reagents and Chemicals

Human Vascular Smooth Muscle (HVSMC), RAW mouse macrophages were purchased from American Type Cell Collection (ATCC), Dulbeccos Modified Eagles Medium (DMEM), HAM F12 medium, Endothelial growth factor, Penicillin, Streptomycin, TES, L-Ascorbic Acid, Nicotenamide Adenine Dinucleotide (NAD), Lipoic Acid, Trolox, Probucol, sodium Chloride Dimethly Sulphoxide (DMSO), MTT (3-[4,5-dimethylthiazol-Z-yl]-2,5-diphenyltetrazolium), Bradford reagent, Octanal, Triton X-100, Tryphan Blue, Trypsin, Bovine serum Albumin (BSA), Agarose, Streptozotocin, Phosphate buffered Saline (PBS), Sodium dodecyl sulphate (SDS) were purchased from SIGMA Chemicals Co. (St Louis, MO, USA). Carboxy-2', 7'-dichlorodihydrofluroscein diacetate (CM-H_2DCFDA) was purchased from Molecular probes Inc (Eugene, OR, U.S.A). Ficoll–Paque Plus, Rediprime II Random prime Labeling System, Nitrocellulose membrane Hybond N+, G-50 microspin columns were purchased from Amesham GE Health Sciences. QIAquick PCR purification Kit, Taq PCR Master Mix Kit, Omniscript Reverse Transcription Kit were purchased from Qiagen (U.S.A). Primers were synthesized by Bioserve–Biotechnologies India Pvt Ltd. Cytokine ELISA Kits (Duo Set ELISA Developing System) and reagent substrates were purchased from R&D Systems, TRIZOL reagent was purchased from Invitrogen Life Technologies, H_2O_2 and D-glucose was purchased from Qualigens. Fetal Bovine Serum was purchased from Hyclone. Tissue Culture Plasticware and cell scrapers were purchased from NUNC.

2.2 Cell Culture

Cell lines were obtained from ATCC and immediately culture was initiated in DMEM culture medium supplemented with 10% FBS, Penicillin (100mg/lit) and Streptomycin (60mg/lit) in a T-25 cm^2 tissue culture flask. Cells were incubated at 37^0C in a humidified 5% CO_2 incubator. Once the cells reached 70-80% confluence, the medium was removed and washed with PBS. Using a sterile cell scraper the cells were scrapped gently to one corner of the flask containing culture medium. Using a sterile pipette, the cells were transferred to T-75 cm^2 tissues culture flask. The flask was incubated at 37^0C and observed for cell growth under an inverted microscope.

2.2.1 Thawing of cells

Frozen cells were rapidly thawed by incubating the vials in 37^0C water bath. As soon as the cells were thawed, they were removed from the water bath and decontaminated by dipping in 70% alcohol. Using sterile pipette the cells were transferred to 5ml of culture medium in 15ml centrifuge tube. The cells were centrifuged at 1500rpm for 5min.The supernatant was gently decanted without disturbing the cells. The cell pellet was resuspended in 5ml of culture medium and transferred to a T-25 cm^2 tissue culture flask. The flask was incubated at 37^0C in 5% CO_2 incubator and observed for cell growth under inverted microscope.

2.2.2 Cell Preparation for experiment

For the experiment purpose logarithmically growing cells in culture medium at 70-80% confluence were used. Cells passaged between 3 to 8 generation were used in experiments. Cells were harvested from the flask by trypsinization or cell scrapper, washed and resuspended in fresh culture medium. After performing the cell count by tryphan blue method, required number of cells were seeded into flask, six well plate, microtitre plates or chamber slides based on the experiment to be conducted. In all the experiments the cells were treated with drugs or the dye and the final concentration of DMSO did not exceed 0.1%. The control cells were treated with only vehicle (DMSO) for comparison.

2.3 Assay for ROS production

2.3.1 Treatment with H_2O_2:

CM-H_2DCF-DA, a cell permeable fluorogeneic probe that is useful for the detection of ROS, was used to measure the degree of ROS accumulation within the cells. Cells were seeded in 96 well plates at density of 5000 cells/well in the regular medium and allowed to grow to 60-70% confluency. Cultured cells were briefly washed once with PBS and further incubated in the medium without FBS containing 20µM of CM-H_2DCF-DA dye and incubated at 37^0C for 30 min. CM-H_2DCF-DA is non fluorescent permeable molecule which is acted upon by intracellular esterase to produce H_2DCF. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to

the highly fluorescent 2',7'-dichloroflurescein (DCF) and emits fluorescence. Cells were washed twice with PBS and treated with PBS containing different concentrations of freshly prepared H_2O_2 in dark condition. Plates were placed immediately in a fluorescent micro plate reader (Gemini Spectromax EM, Molecular Devices) at 37^0C. Fluorescence was monitored continuously for 90min using excitation wavelength at 485nm and emission at 530nm. In each experiment, fluorescence increase was measured in 12 replicated cultures for every concentration of H_2O_2**.** DCFH with culture medium was used as blank control (Hsu and Wen, 2002).

2.3.2 Treatment with Glucose:

Cells were seeded in a 96well micro plate and allowed to grow to 60-70% confluence. The culture medium was removed and cells were further incubated in different concentrations of glucose for 24hrs. The medium was removed and cells were washed with PBS and further incubated in medium containing 20µM CM-H_2DCF-DA dye for 30min at 37^0C in CO_2 incubator. The dye was removed, cells were washed with PBS and glucose induced fluorescence was measured as mentioned above. CM-H_2DCF-DA dye was freshly prepared in DMSO for each experiment.

2.4 Antioxidant treatment on ROS production

2.4.1 Treatment on H_2O_2 induced ROS production:

Cultured cells were allowed to grow up to 60-70% confluency. The cells were preincubated for 2hrs in culture medium containing L-Ascorbic acid (antioxidant). The cells were washed with PBS and further incubated in 20µM CM-H_2DCF-DA dye for 30min at 37^0C. Cells were washed with PBS and treated with 100µM H_2O_2.The increase in fluorescence was measured as described above. Otherwise the amount of ROS induced fluorescence under various treatment conditions were captured under a fluorescent inverted microscope equipped with a cool SNAP-pro color digital camera.

2.4.2 Treatment on glucose induced ROS production:

Cultured cells were allowed to grow up to 60-70% confluency. The cells were incubated in 100mM of glucose for 24hrs followed by further incubation with antioxidant, insulin and troglitazone (antidiabetic drug) for 2hrs at 37^0C. The cells were washed with PBS and incubated for 30min with CM-H_2DCF-DA dye. The increase in fluorescence was measured as described earlier. Otherwise the amount of ROS production under various treatment conditions were instantly captured under a fluorescent inverted microscope equipped with a cool SNAP-pro color digital camera.

2.4.3 Measurement of TNF α induced Intracellular ROS generation:

TNFα induced intracellular ROS was measured by a fluorometer, via the method of detecting the oxidation of CM-DCF-DA dye as described earlier. Macrophages upon reaching confluency were incubated with different concentrations of human TNFα recombinant protein for 12hrs and then incubated with insulin or ascorbic acid for 2hrs.The cells were washed and incubated with 20µM CM-H_2DCF-DA dye for 30min in serum free medium at 37^0C. The relative fluorescence intensity was detected at an excitation wavelength of 485nm and an emission wavelength of 530nm (Hsu and Twu, 2000).

2.5 Cell Viability Assay (MTT Assay)

The MTT assay is used to measure the cell viability. The principle of this assay is that the compound 3-(4, 5, dimethyl thiazol-2-yl)-2, 5 diphenyltetrozolium bromide (MTT) undergoes cellular reduction by the mitochondrial dehydrogenase of viable cells into blue formazan that can be measured spectrophotometrically. Cells were seeded in a 96well plate and allowed to grow to 60-70% confluence. The culture medium was removed and cells were incubated with various concentrations of glucose for various time intervals as indicated in the results. At the end of incubation, MTT at (1mg/ml prepared in PBS) was added to each well and incubated at 37^0C for further 2-3hrs. The medium was removed gently and DMSO was added to each well in order to solubilze the formazan crystals and kept for mild shaking in

dark. The solubilzed blue formazan in DMSO was quantified with a spectrophotometer at wavelength 540nm. There is a linear relationship between the formazan crystals formed and the number of viable cells present (Meilhac et al., 2000).

2.6 Measurement of cytokine levels in RAW macrophages

2.6.1 Detection of TNFα in the culture supernatant by ELISA:

The secretion of cytokines by various concentrations of glucose in the presence or absence of insulin was measured in mouse macrophages. The RAW macrophages were seeded in a 24 well plate in DMEM+10% FBS medium and incubated at 37^0C. Once the cells reached 70% confluence the cells were treated with various concentrations of glucose and glucose + insulin for 24hrs. Cell supernatant was collected and centrifuged at 1500rpm for 5min to remove any cells floaters. Quantitative detection of TNFα in conditioned media was performed using a specific enzyme linked immunosorbent assay (ELISA) from R& D systems, Minneapolis MN using the manufactures suggested directions. Known concentrations of TNFα were used to generate the standard curve. Streptavidin-horseradish peroxidase served as the detection system. For each experiment, quadruplet's samples were measured. Data represented as the mean (pg/ml) ± S.E. The assay was linear between 31.25pg/ml and 2000 pg/ml.

2.6.2 Detection of MCP-1 and MMP9 cytokines in primary human blood macrophages:

Fresh human macrophages were obtained from healthy donors using an approved institutional review board protocol and isolated as described. Human whole blood was collected into a sterile 15ml centrifuge tube containing heparin (10IU/ml) and gently mixed to avoid any clotting of the blood. The blood was diluted 1:1 in sterile PBS in a laminar hood and overlaid onto ficoll already present in another 15ml sterile centrifuge tube very gently so that blood stays for longer time above the ficoll. The ficoll and blood are added in equal ratios (1:1). The samples are centrifuged at 1500rpm for 30min at room temperature. After centrifugation the white interphase between the plasma fraction and the ficoll fraction was taken out gently with a sterile pipette into fresh tube. The cells were washed with PBS/medium followed by centrifugation at 1500rpm for 10min each twice.

The supernatant was discarded and cells were resuspended in fresh culture medium and cell count was done with crystal violet. Cells were seeded at a density of 10 x 10^5 cells /well in a 6 well plate and incubated at 37^0C in 5% CO_2 incubator. Macrophages were allowed to adhere for 3hrs and non adherent lymphocytes were washed away to get a nearly pure macrophage culture and incubated in fresh culture medium overnight. The medium was removed and cells were incubated under various glucose conditions and glucose + insulin and antioxidant for 24hrs at 37^0C in 5% CO_2 incubator. Cell supernatant was taken and cytokine levels were estimated by

ELISA according to manufacturer's protocol (R& D systems, Minneapolis MN) as described above.

2.7 Total RNA extraction and Northern Blot analysis

Total cellular RNA from tissues and cells was isolated using the Trizol reagent. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987).

2.7.1 Extraction from Tissues

The tissue samples were homogenized in 1 ml of TRIZOL reagent per 50-100 mg of tissue using a glass-Teflon or power homogenizer (Polytron). The sample volume never exceeded 10% of the volume of TRIZOL reagent used for homogenization.

2.7.2 Cells Grown in Monolayer

The cells were directly lysed in a culture dish by adding 1 ml of TRIZOL reagent to a 3.5cm diameter dish and passing the cell lysate several times through a pipette. The amount of TRIZOL reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. An insufficient

amount of TRIZOL reagent will result in contamination of the isolated RNA with DNA.

2.7.3 PHASE SEPARATION

The homogenized samples were incubated for 5 minutes at 15 to 30^0C to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml of TRIZOL reagent. The tubes were shaken vigorously by hand for 15 seconds and incubated at 15 to 30^0C for 2 to 3 minutes. The samples were centrifuged at no more than 12,000× *g* for 15 minutes at 2 to 8^0C. Following centrifugation, the mixture gets separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL reagent used for homogenization.

2.7.4 RNA PRECIPITATION

The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. 0.5 ml of isopropyl alcohol was added per 1 ml of TRIZOL reagent used for the initial homogenization. The samples were incubated at 15 to 30^0C for 10 minutes and centrifuged at no more than 12,000 × *g* for 10 minutes at 2 to 8^0C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

2.7.5 RNA WASH

The supernatant was removed and RNA pellet was washed once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The samples were mixed by vortexing and centrifuged at no more than 7,500 × *g* for 5 minutes at 2 to 8^0C.

2.7.6 REDISSOLVING THE RNA

At the end of the procedure, briefly the pellet was dried (air-dry or vacuum-dry for 5-10 minutes). It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60^0C. For northern blot analysis, 10µg of total RNA was denatured in formamide and formaldehyde RNA loading buffer at 60^0C for 5min and chilled on ice for 10min. The RNA was separated by electrophoresis on 1.2% w/v agarose gels containing formaldehyde. A gel running buffer was used as the electrode buffer. Electrophoresis was carried at 80V until the 28s and 18s RNA got separated well or the tracking dye has reached 3cm before the end of the gel.

2.7.7 Transfer of Denatured RNA to Nitrocellulose Filters:

Glyoxylated RNA was transferred immediately after electrophoresis from agarose gels to nitrocellulose membrane (Hybond N+-membrane, GE Health Care Life Sciences) by using the vacuum transfer apparatus (Vaccu gel XL, GE Health Care Life Sciences) by using the following protocol.

Before use, the mask should be cut to make an opening or window of the gel. To achieve sealing of the gel, it is important that the window is slightly smaller than the gel. The gel should overlap the window by between 3-10mm.It is recommended that the overlap should not exceed 10mm since the gel may float upwards when the solution is applied, giving a poor seal. For vacuum blotting either nylon, PVDF or a nitrocellulose transfer membrane, with a pore size of 0.45mm or smaller, is used.

The gel size was measured and the membrane was cut little bigger and pretreated by wetting in 20xSSC buffer for 20minutes. The vacuum blot was cleaned, dried and connected with the vacuum pump and in line liquid trap connected. The porous support screen was pretreated in distilled water and placed on the inner rim of the base unit with the shiny side up. The pretreated membrane was positioned under the mask so that it covers the window in the plastic mask completely and no air bubbles are trapped in between the membrane and the porous support system. The gel was slided very gently from the support frame on to the membrane to fill the window. The vacuum pump was switched on to immobilize the gel and immediately solution I (depurination solution) was poured on the centre of the gel. Sufficient solution was poured to cover the gel and left it for 15 min. During

the depurination steps the vacuum was stabilized at 60 mbar. After 15min the blotting unit was tilted using the collapsible stand and the residual liquid was removed by pipette. Solution II, denaturation solution, was poured using enough solution to cover the gel surface for 30min and then removed completely as before. Solution III, neutralization solution was poured as before. Using enough solution to cover the gel surface for 30min and then removed completely as before. Solution IV, Transfer Solution was poured to cover the gel to about twice its depth and left for 60min. With the vacuum still on, corner of the gel was lifted slowly, leaving the membrane in place. The vacuum was switched off; the membrane was removed and blotted in between filters papers until it is completely dry. The dried membrane was placed in a UV crosslinker (UVC 500- Hoefer) for 5 min at 1500x100μJ/Cm^2 for efficient binding of RNA to the membrane. The membrane was incubated in pre-hybridization buffer at 42^0C for 12-16 hrs in hybridization chamber.

2.7.8 Preparation of cDNA probes:

Specific DNA probes were labeled with (α-^{32}P)-dCTP by random priming using the Rediprime kit (Amersham Biosciences) according to the following protocol: The DNA of the specific genes to be checked were diluted to a labelled concentration of 2.5-25ng in 45ul of 10mM Tri HCl pH 8.0, 1mm EDTA (TE buffer). Sample was denatured by heating to 95-100^0C for 5 minutes in a boiling water bath and snap cooled on ice for 5 minutes after denaturation. The sample is

centrifuged briefly to bring the contents to the bottom of the tube. The denatured DNA was added to the reaction tube and 5µl of [^{32}P]-dCTP was added and mixed by pipettnig up and down for about 12 times, moving the pipette tip around in the solution. The tube is incubated at 37^{0}C for 15 minutes and the solution purified through the G-50 radioactive purification column. The flow through containing only the labelled DNA probe was denatured by heating to 95-100^{0}C for 10 minutes in a boiling water bath. The probe was snap cooled on ice for 10 min and centrifuged briefly to bring the contents to bottom of the tube. The labelled probe was added to the membrane and incubated in fresh hybridization buffer at 42^{0}C for 24hrs.

2.7.9 Purification of the labeled DNA probe:

Purification of the radiolabelled probe was done by using G50 spin columns (Amersham Biosciences). Briefly a G50 column was taken, bottom of the column was broken and placed into a 1.5ml microfuge tube. The column was centrifuged at 3000rpm for 1min at room temperature in order to allow the column bed to be formed. The column was transferred to a fresh 1.5ml microfuge tube and at the top of the column the radiolabelled DNA probe solution is overlaid very gently without disturbing the beads. The column was centrifuged at 3000rpm for 1min, the flow through was collected and the column was discarded very carefully in the radioactive waste container.

2.7.10 Washing of the membrane:

Membrane was washed in 1x SSC, 0.5% (w/v) SDS for 30min at 42^0C, followed by another wash in 0.1% SSC, 0.5%(w/v) SDS for 15min at 50^0C. The membrane was washed in 0.1x SSC for 5min and wrapped in saran wrap and exposed to X-ray film (Kodak) in cassette. The cassette was kept at -80^0C overnight. Next day x-ray was developed for the presence of bands. Blots were stripped and rehybridized with [α-^{32}P]-dCTP labeled β-actin in order to normalize the signal.

2.8 Reverse Transcriptase and PCR

Total RNA was extracted by the Trizol method as described above from tissues and cells. 1µg of RNA was reverse transcribed for 60 min at 37^0 C using the Omniscript transcription cDNA synthesis kit (Qiagen) in the presence of oligo (dt) 15 primer.

Example of a reaction:

10x Reaction Buffer	: 2µl
dNTP mix	: 2µl (5mm each dNTP)
Oligo dt prime	: 2µl (10µM)
Omni script RT	: 1µl (4U/µl)
RNase inhibitor	: 2µl (5U/µl)
RNA	: 1µg
RNase free water	: Variable
Total volume	: 20µl

Cycle conditions:

25^0 C: 10min

37^0 C: 60min

95^0 C: 5min

Quantitative PCR was performed by using the Taq PCR mastemix kit (Qiagen). PCR primers for each gene were designed by using Primer 3.0 software. Each PCR was carried out in a 20µl volume for 95^0 C for 5min for initial denaturation, followed by 40 cycles at 95^0 C for 30sec, 50^0 C for 60sec and 72^0 C for 60sec, followed by a final extension of 72^0 C for 10min. The PCR samples were separated on 1% DNA agarose gel and gene modulation was captured under UV light. Value of each gene was normalized to expression level of β-actin mRNA level. Results were expressed as fold increase to the control, which was arbitrary assigned a value of 1x.

Primers sequence used to quantify mRNA by northern blot and RT-PCR were designed using Primer 3.0 software. Oligonucleotides used were as follows: mouse ***FALDH*** sense 5'-TGAAGCATCCCTCCAAAATC-3', and antisense, 5'-GTTGG GGCTATGTAGCGTGT-3', mouse ***Catalase*** sense 5'-ACACCTTCAAGTTGGTT AAT-3', and antisense, 5'-ATAAGAGG GTAGTCCTTGTG-3' and ***β-actin*** sense 5'-TTGTCACCAACTGGACGATATGGA-3', and antisense 5'- GATCTTGTCTTC ATGGTGCTAGG-3'.

2.9 FALDH enzyme assay

2.9.1 Preparation of microsomal subcellular fraction:

Animals were anesthetized and sacrificed by cervical dislocation; the livers were quickly removed and placed in ice-cold (4^0 C) 0.25M sucrose. All subsequent procedures for processing the microsomal fraction were carried out at 4^0 C. Liver homogenates (10%w/v) were prepared in 0.25M sucrose using a Teflon pestle and mechanical homogenizer. The homogenate was centrifuged at 7000g for 15min, with the resulting pellet discarded and supernatant retained for centrifugation at 19000g for 5min. The 19000g supernatant was centrifuged at 100,000g for 60min to obtain the microsomal pellet. The pellet was resuspended in 10mM potassium phosphate buffer, pH 7.4, containing 2mm mercaptoethanol and protease inhibitors(Roche) and was designated as the crude microsomal fraction**.** Protein concentrations were determined with commercial Bradford reagent (Sigma) (Mitchell and Petersen, 1989).

2.9.2 Enzyme Assay:

The enzymatic oxidation of aldehydes by FALDH was monitored spectrophotometrically by measuring the production of NADH at wavelength of 340nm. The specific activity was determined at 37^0 C, in a 1.0ml reaction mixture containing of 100mM phosphate buffer (pH7.4), 1.0mM NAD^+, 2.0mM pyrozole, 100µg for crude microsome protein and 0.25% of Triton X 100. Triton x-100 was

used to solubilze the microsomal membranes. Each sample was incubated at 37^0 C for 5min and the reaction was initiated by adding 5µl of 100mM octanal solution in ethanol to achieve a final aldehyde concentration of 500µM. Control incubation consisted of identical reaction mixture, but the reaction was initiated by addition of ethanol lacking octanal. The reaction was typically monitored for 5min using a chart recorder. The choice of cofactor for the enzymatic analysis was based on the fact that FALDH mediated oxidation of aldehyde in the presence of NAD^+ resulted in higher Vmax values that those observed using $NADP^+$. Pyrazole was added to the reaction mixture to inhibit alcohol dehydrogenase enzyme (FADH). FALDH dependent enzyme activity was calculated as compared to the normal control tissue samples.

2.10 Animal treatment and protocol

2.10.1 Streptozotocin (STZ) Mice:

Male (BALBc) mice of 6-8 week of age were obtained from in-house animal facility at Discovery Research, DRL Miyapur. Mice were feed a pelleted rodent chow diet (NIN – Hyderabad) for one week before initiation of studies. Mice were maintained in a temperature controlled (25^0 C) facility with a strict 12hrs light /dark cycle and given free access to food and water. Streptozotocin was dissolved in sterile citrate buffer and injected intra peritoneally into the mice (100mg/kg) in 300µl within 5min of preparation to overnight fasted animals. Control animals were given an equal volume of citrate buffer. After STZ injection the animals were given free

access to water and food. Consumption of water was measured everyday for the first 10 days because increase in water consumption is an indication of onset of diabetes and destruction of pancreas. After 15 days of STZ treatment plasma glucose was estimated. Mice with high glucose (400-500mg/dl) were divided into two groups of five animals each, one group was STZ only, second group was STZ + L- Ascorbic acid and third group was normal control BALBc mice without any STZ treatment. L-ascorbic acid was given at a dose of 800mg/kg in drinking water for additional 15 days. After the treatment the mice were sacrificed, liver and thoracic aorta were collected and processed for the preparation of total RNA or liver microsomes.

2.10.2 *db/db* Mice:

The *db/db* mice have an autosomal mutation in the leptin receptor on chromosome 4, which is required for the leptin mediated metabolic pathway of energy expenditure. The *db/db* mice exhibited severe diabetic phenotype with marked hyperglycemia, hyperphagia and decreased life span. These animals exhibit significant hyperinsulinemia in the first few months of life and are transient with β-cell failure exemplified by hyperplasia and hypertrophy of the β-cells. *db/db* mice were obtained from Jackson laboratories (Bar herbor, ME) at the age of 6 weeks and was maintained on feed 5K52 (Labdiet) with free access to water upto an age of 12 weeks when the animals are on diabetic situation. Animals were euthanized by cervical dislocation and liver , aorta was rapidly collected for RNA isolation and microsome preparation.

2.10.3 ZDF (Zucker Diabetic Fatty Rats):

ZDF have an autosomal recessive mutation in the leptin receptor and has a defect in the pancreatic β-cell that effects the insulin production which later progresses to state of insulin deficiency. The ZDF rats develop overt diabetes with severe hyperglycemia, polyuria and poydipsia similar to human type 2 diabetes. The ZDF rat is a good type 2 diabetic model and used extensively for testing small molecule antidiabetic compounds. The rats were obtained from Charles – River Inc at the age of 6 weeks. The animals were fed on Purina 5008 diet obtained from Charles – River Inc for 12 weeks until they develop full diabetes with glucose (400mg/dl). The animals were sacrificed and the liver was collected rapidly for further studies.

2.10.4 Diet Induced Obesity (DIO) Mice:

C57BL/6J mice were obtained from our in-house animal facility at Discovery Research, DRL Miyapur at the age of 6 weeks and feed on high fat diet D12492-with 60% Kcal Fat (research diets) for upto 3 months (18 weeks). The body weights and the lipid parameters were checked every week during 3 months. The animals were sacrificed and the liver was collected for further studies.

2.10.5 Cholesterol Fed Rats (CFR):

Sprague- Dawley rats, 225-300gms were taken from the inhouse animal facility and feed on high fat diet (2% cholesterol, 1% cholic acid, 20% dalda, 6%

coconut oil, 71% NIN powder feed) for 10 days .When the animals showed dyslipidemia (approx one week) the animal were sacrificed and the liver was rapidly collected further studies.

2.11 Proteomics

2.11.1 Sample Preparation:

The RAW 264.7 macrophage cells were grown in complete medium in T-25 flask till they reached 70% confluence. The cells were treated with various treatment conditions as described in the earlier sections for 24 hrs. After the incubation time macrophages were washed with PBS, scraped off the flasks, and collected by centrifugation. 100mg of cell pellet was washed twice in buffer R1 (40mM Tris pH 7.4) with protease inhibitors. Pellet was homogenized in 500µl of buffer R1 containing protease inhibitors and 2mM PMSF. After 15 min at 4^0 C the tubes were centrifuged at 13000xg for 15min. The supernatant was discarded and the pellet was resuspended in 300µl of buffer 2 (8M urea,4% CHAPS, 2mM TBP, Ampholyte 0.2%, 40mM Tris and bromo phenol blue 0.002%). The tubes were vortex (15sec) and centrifuged at 13000xg for 15min. The supernatant was stored at -80^0 C and used as protein source. Protein concentration was determined with commercial Bradford reagent (Sigma).

2.11.2 2-Dimensional Gel Electrophoresis (2-DE):

2-DE was performed in an Etton IPGphor IEF system (Amersham Pharmacia Biotech) using 17cm, pH 3-10 IPG strips. 200µg of protein was applied to pH 3-10 IPG strips during rehydration at 20^0 C for 10hrs. IEF was carried out at 20^0 C at 50mA/strip, 150V for 2hrs, 500V for 1hr and 8000V until 70,000V accumulated (approximately 15hrs) according to manufacturers instructions.

After IEF, strips were equilibrated with shaking for 15min in 3ml of 50mM Tris-HCl, 6M urea, 2% w/v SDS, 30% v/v glycerol and 0.1% w/v bromo phenol blue containing 65mM DTT, followed by an additional 15min in the same solution with 100mM iodoacetamide instead of DTT. SDS-PAGE was carried out on vertical DALTsix system (Amersham Biosciences) with 12.5%, 3% polyacrylamide gels. Using a gradient marker the gel was poured until a desired height was reached and allowed to polymerize for at least 1hour. The strip was blotted gently and loaded along with molecular weight marker (spotted on a application paper) onto the gel, sealed with 1% agarose in electrophoresis buffer and the gel was run at 200V for 8-9hrs. All reagents were of plus one grade purchased from Amersham Biosciences.

Gel staining was performed accordingly on a rotatory shaker with gentle agitation. The gel was placed in a glass tray containing fixing solution (40% ethanol, 10% acetic acid) and agitated on a shaker for at least 1 hour. The fixing solution was drained out gently and the gel was incubated in sensitizing solution (30% ethanol, 0.2% sodium thiosulphate, 6.8% sodium acetate) and agitated for 30min. The gel

was washed in double distilled water 3 times of 5min each and incubated in 0.25% silver nitrate solution for 20min and washed with double distilled water 2 times of 1 min each. Developing solution was added to the gel (2.5% sodium carbonate, 0.015% formaldehyde) and agitated gently until yellow or brown precipitate appeared; agitation was continued until desired intensity of spot was achieved. The developing solution was removed and the stopping solution (5% acetic acid) was added and agitated for 10min. The gel was washed with double distilled water 3 times of 5min each, stained gels were digitized with an image scanner and analyzed with PD QUEST software. The reproducibility of 2-DE was evaluated by preparing three replicates of protein from treatment.

2.11.3 MALDI-TOF MS:

Spots which showed an increase or decrease in volume by two fold or more as compared to the control were excised, and cut into 1-2mm pieces. These were added to 100 µL 25mM NH_4HCO_3/50% ACN, incubated for 10 min to remove CBB, then rinsed thoroughly. The gel pieces were then dehydrated and dried thoroughly in a vacuum centrifuge for a few minutes. The dried gel pieces were rehydrated with 20 µL 50mM NH_4HCO_3 (pH 8.0) containing 20 µg/mL trypsin (Promega, Madison, WI, USA) and the proteins in the gel pieces were digested overnight at 37^0 C. After digestion was complete, the supernatant was transferred to another tube, and the gel piece incubated with 20 µL 50% ACN/5% formic acid for 10 min at room temperature. The extract was transferred to the primary supernatant, and the

extraction was repeated once more. The extracted digests were evaporated to dryness in a vacuum centrifuge. The digests were re-dissolved in 2 ml 0.1% TFA. The solution was vortexed and centrifuged. The supernatant (1µL) was mixed with 1 µL 10 mg/ml CHCA in 50% ACN/ 0.1% TFA and the mixed solution was spotted onto a 96-spot MALDI target.

MALDI–TOF analyses were performed on a Voyager DESTR MALDI–TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). The MALDI–TOF mass spectrometer was operated in positive-ion, delayed-extraction (200 ns delay time) reflector mode. Results were analyzed with Data Explorer software (Applied Biosystems) to obtain accurate masses (± 50–100 ppm) for all the peptides in the tryptic digest. The results from the PMF, together with the p*I* and molecular mass values (estimated from 2-DE gels), were used to search the Swiss-Prot or NCBInr protein databases with a special search tool [MS-FIT from Protein Prospector V 4.0.4 (http://prospector.ucsf.edu)], which compares the experimentally determined tryptic peptide masses with theoretical peptide masses calculated for proteins contained in these databases. Search parameters were ± 50– 100 ppm peptide mass tolerance and one maximum missed cleavage.

2.11.4 ZYMOGRAPH:

After IEF, strips were incubated in buffer containing 50mM Tris pH 8.8, 10% glycerol with shaking for 10min and replaced with fresh buffer and incubated for additional 10min.The strips were further incubated in substrate buffer (50mM Tris

pH 8.8, 0.4mg/ml NBT, 0.08mg/ml PMS, 0.4mg/ml NAD/NADP, 0.8% octanal) for 20min in dark at room temperature until a dark blue precipitate formazan band appeared on the strip. The reaction was stopped by incubating the strip in stop solution (7% Acetic acid).The appearance of blue band signifies the activity of FALDH enzyme in the protein sample.

2.12 Statistical Analysis:

Results are expressed as the mean ± S.E of the average responses in multiple experiments (n = 3). Data were analyzed by analysis variance followed by Student's t test for the paired components.

3.1 Hydrogen Peroxide induced Reactive Oxygen Species in Human Vascular Smooth Muscle cells (HVSMCs) and RAW 264.7 Mouse Macrophages:

Hydrogen peroxide a membrane permeable reagent leads to the generation of acute oxidative stress (ROS) .Hydrogen peroxide is widely used to assess the effect of ROS on the modulation of genes like c-fos (Devary et al., 1991), NFκB in various systems (Schreck et al., 1991). Hydrogen peroxide is produced in large quantities by granulocytes and macrophages during the inflammatory process (Cerutti, 1985; Halliwell and Gutteridge, 1990). To investigate whether H_2O_2 leads to the production of ROS in HVSMCs, the effect of increasing concentration of H_2O_2 on ROS generation was investigated. The generation of ROS was measured by the oxidation of carboxy DCF-DA fluorescence dye. Briefly cells were grown in carboxy DCF-DA for 30 min before culturing in media containing various concentrations of H_2O_2 and immediately measured the emission of fluorescence for 90 min. H_2O_2 increased ROS levels significantly in a dose dependent manner in HVSMCs **(Fig 1A).** Since HVSMCs are a primary culture and are difficult to expand and maintain for longer period, further studies were confined to RAW macrophages. To confirm whether RAW macrophages respond in a similar way as HVSMCs, H_2O_2 induced ROS generation was monitored in macrophages using similar experimental conditions. The results clearly indicate that, H_2O_2 increased ROS levels significantly and dose dependently **(Fig 1B).** Treatment of macrophages with L-ascorbic acid, a well known antioxidant, quenched ROS produced by H_2O_2 **(Fig 1C).** This data confirm that

macrophages can be used as model to study oxidative stress. To further confirm the production of ROS by H_2O_2 RAW macrophages were monitored by confocal microscopy for oxidation of carboxy DCF-DA dye in presence various concentrations of H_2O_2 and emission of fluorescence was observed. As shown in (**Fig 1D)** the fluorescence signal intensity increased with increasing concentrations of H_2O_2. The intensity of fluorescence emission was reduced to a basal level when cells were treated with ascorbic acid **(Fig 1D)**.

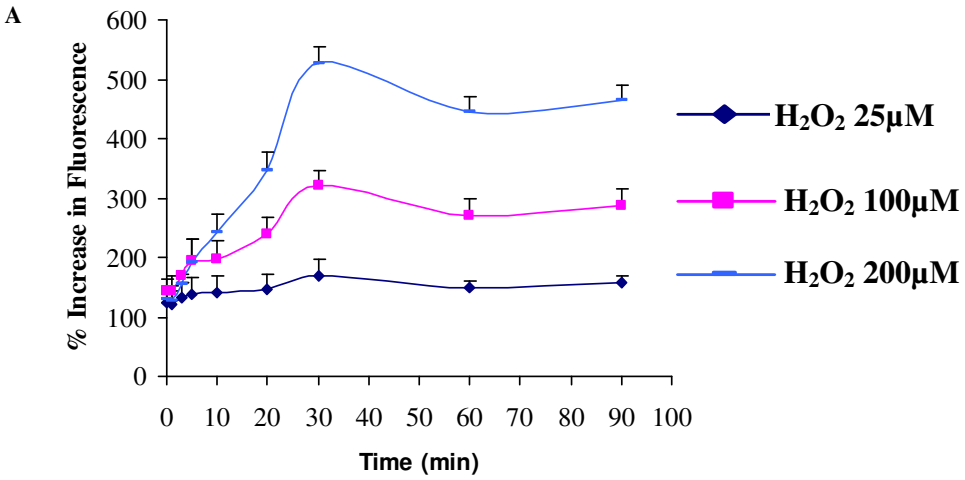
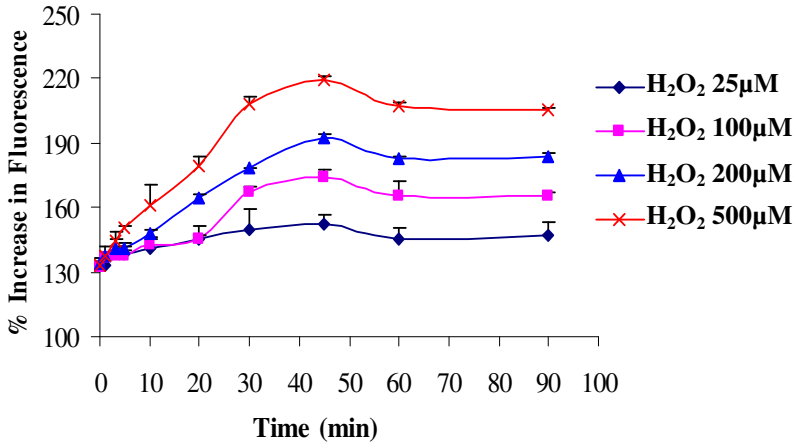


Figure 1. Increase in oxidative stress induced by Hydrogen Peroxide in HVSMCs: (A), Human Vascular Smooth Muscle Cells (HVSMCs) were treated with various concentrations of H_2O_2 as described in "Experimental procedures" and generation of ROS was measured by fluorescence emissions due to oxidation of Carboxy-DCF-DA dye. Results are expressed as a mean ± S.D. from 3 independent experiments.

B



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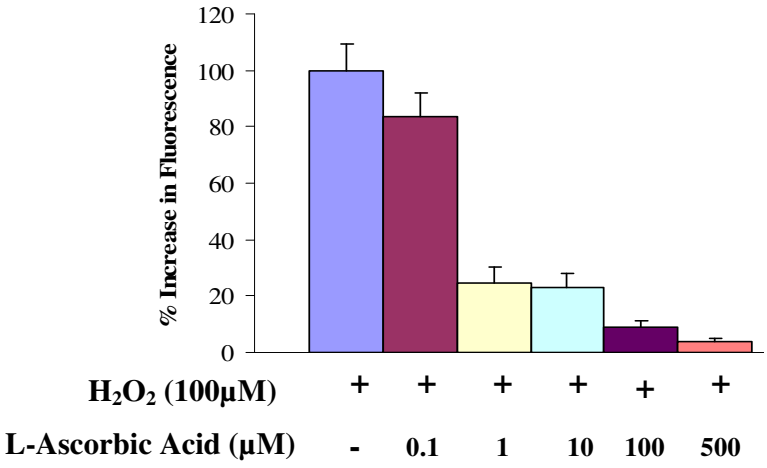


Figure 1. Increase in oxidative stress induced by Hydrogen Peroxide in RAW macrophages: (B) Macrophages were treated with various concentrations of H_2O_2 as described in "Experimental procedures" and generation of ROS was measured by fluorescence emissions due to oxidation of Carboxy-DCF-DA dye. Results are expressed as a mean ± S.D. from 3 independent experiments. (**C**), RAW 264.7 macropahges were treated with 100µM H_2O_2 alone or 100µM H_2O_2 plus various concentrations of ascorbic acid. After 30 mins generation of ROS was measured by fluorescence emissions due to oxidation of carboxy-DCF-DA dye. Data are mean ± S.D, n = 12.

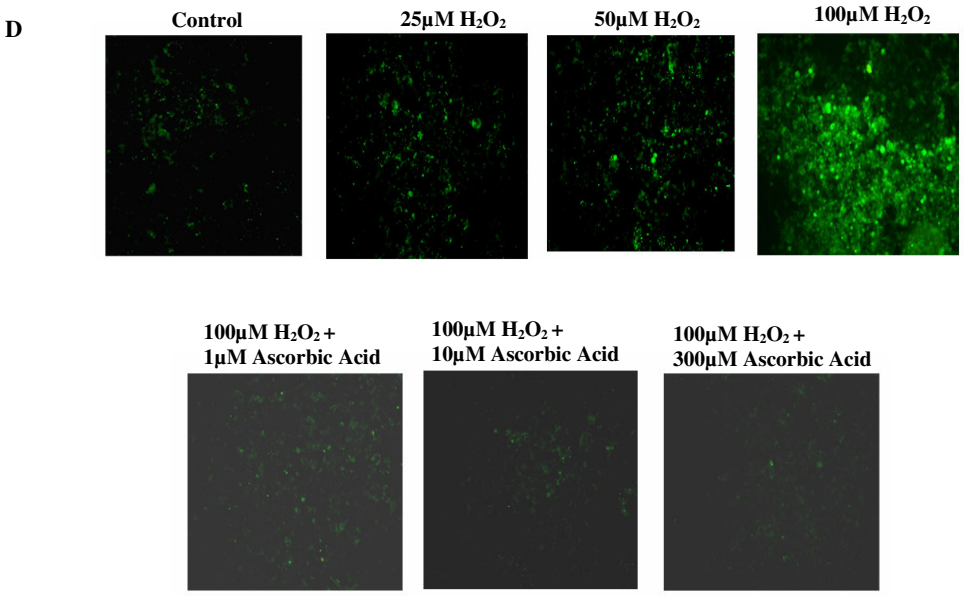


Figure 1. Increase in oxidative stress induced by Hydrogen Peroxide: (**D)** Dichlorofluorescein (DCF) fluorescence from RAW 264.7 mouse macrophages cells was imaged with digital camera attached to the inverted fluorescent microscope. Cells were treated with H_2O_2 and/or with ascorbic acid.

3.2 Hyperglycemia increased Reactive Oxygen Species in Macrophages:

It has been observed that in many cell types (such as endothelial cells, adipocytes and vascular smooth muscle cells) hyperglycemia induces the generation of reactive oxygen species (ROS) which affects their normal functioning. ROS is also known to be one of the causative factors for the development of atherosclerosis in diabetic patients. However, very little is known about the molecular mechanism of how diabetes induced ROS affect macrophages which leads to the development of atherosclerosis. Therefore the effect of hyperglycemia induced ROS in macrophages was examined, since macrophages are the cells that generate ROS upon stress,

release immune mediators and uptake oxidized-LDL, which collectively contribute to atherosclerosis.

To investigate whether hyperglycemic conditions increase oxidative stress, the effect of high glucose concentration on the generation of ROS in mouse macrophage cell line, RAW 264.7 was measured. The generation of ROS was measured using carboxy DCF-DA dye, where increased amount of ROS generation results in higher level of fluorescence. RAW cells were grown in media containing various concentrations of glucose for 24 hrs before growing them in media containing carboxy DCF-DA dye for 30 min. The amount of ROS generated under hyperglycemic conditions were measured by fluorescence and results were expressed as the percentage of ROS generated over basal level [basal level of 25mM glucose (NG) = 100%]. Exposure of macrophages to increasing concentration of glucose significantly increased the amount of ROS generation that increased in a dose dependent fashion (**Fig 2A**). Therefore subsequent experiments for hyperglycemic conditions were carried out with 100mM glucose concentration referred to as "high glucose". Treatment of cells with antioxidants, such as lipoic acid, ascorbic acid and probucol before detecting ROS (as mentioned in materials and methods) quenched ROS **(Fig 2B)**.

To further show that hyperglycemia induces ROS generation in macrophages, RAW cells were treated with increasing glucose concentrations and whole cells were monitored by confocal microscopy for oxidation of carboxy DCF-DA dye and emission of fluorescence (as described in materials and methods).

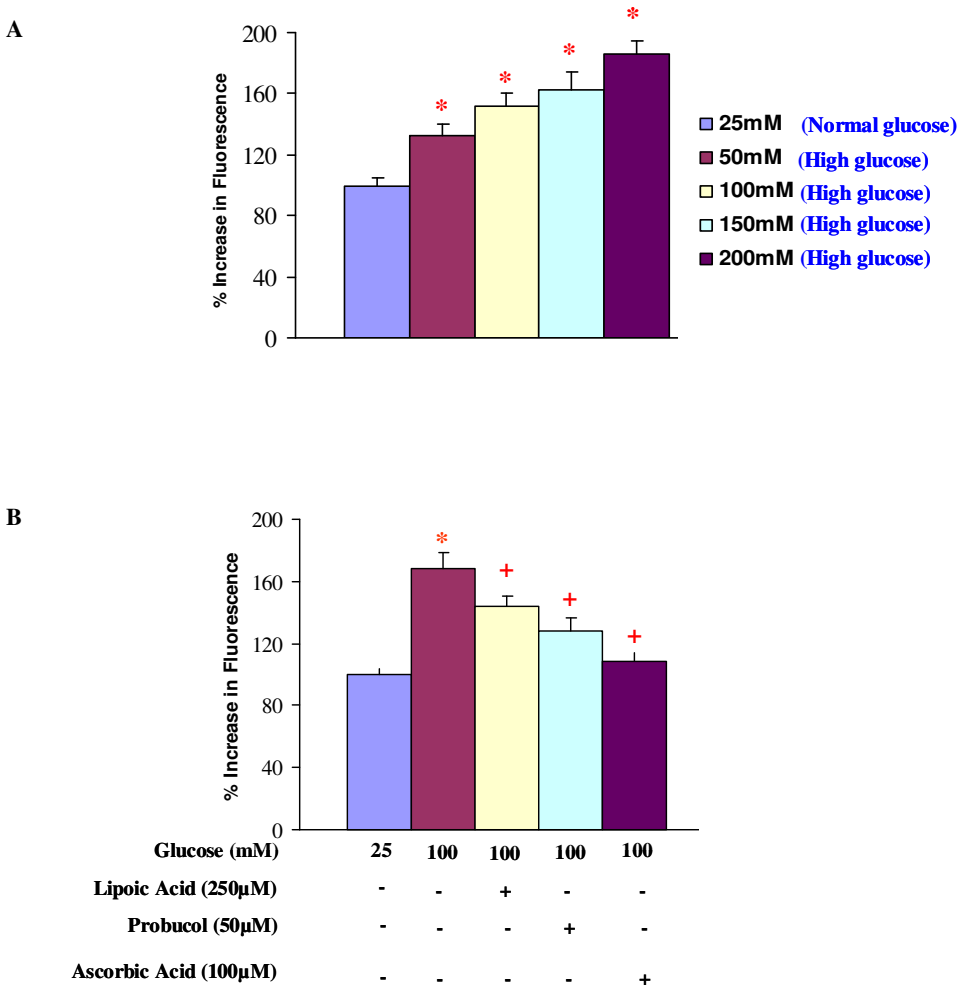


Figure 2. Increased oxidative stress induced in macrophages by hyperglycemia: (A), RAW 264.7 macrophages were cultured in increasing concentration of glucose for 24 h. Intracellular ROS was measured as described under "Experimental procedure". Data are mean ± S.D, n = 12 from 3 independent experiments. Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$). **(B)** RAW macrophages were cultured in 100mM glucose (high glucose) alone or 100mM glucose plus 250µM Lipoic acid, 50µM Probucol or 100µM Ascorbic Acid (AA) for 24 h. Intracellular ROS was determined. Data are mean ± S.D, n = 12. Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$). Plus (+) indicates significant difference over 100mM glucose (High Glucose) treatment alone ($p \leq 0.005$).

As shown in (**Fig 2C**), DCF-DA fluorescence signal intensity increased with increasing concentration of glucose. The amounts of oxidation of DCF-DA dye reflected by intensity of fluorescence emission were reduced to a basal level when cells were treated with various antioxidants such as troglitazone, ascorbic acid and insulin **(Fig 2C)**.

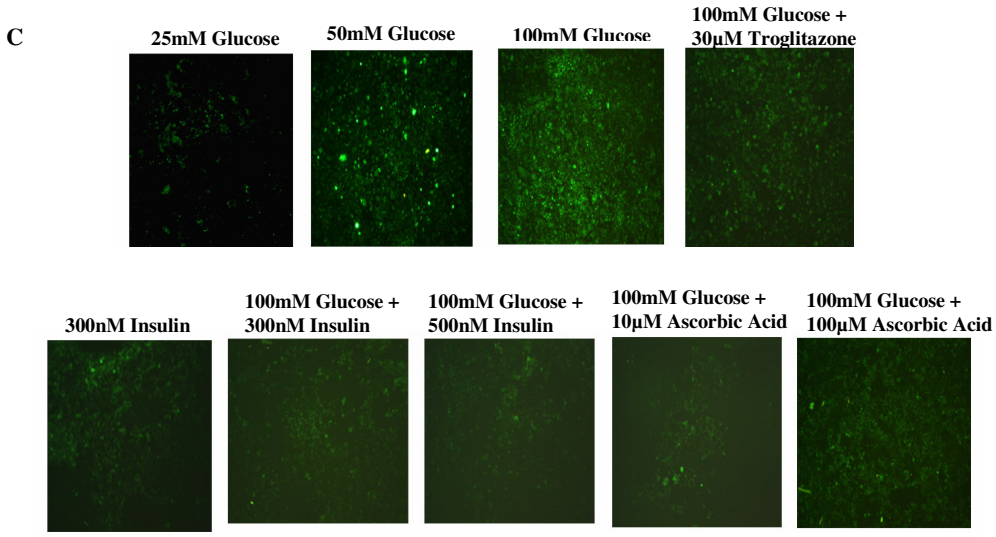


Figure 2. Increased oxidative stress induced in macrophages by hyperglycemia: (C), Dichlorofluorescein (DCF) fluorescence from RAW macrophages was imaged with digital camera attached to the inverted fluorescent microscope. Cells were treated with 100mM glucose and/or insulin, ascorbic acid, troglitazone for 24 h.

3.3 Effect of high glucose on cell viability: Increased oxidative stress due to hyperglycemia activates signaling pathways leading to cell death. To investigate the effect of high glucose concentrations on macrophage survival, MTT cell viability assay was done. Briefly macrophages were treated with glucose at final

concentration of 50, 100, 150 and 200mM in cultures for varying time periods. Cells treated with 25mM glucose were used as controls. There was no significant decrease in cell viability in the cells exposed to 50mM and 100mM until 24 hrs, but a significant decrease in the cells exposed to 150mM and 200mM for 12hrs was observed (**Fig 3).** Therefore in subsequent experiments 100mM glucose was used as high glucose condition because it mimics the hyperglycemic condition observed in diabetic condition.

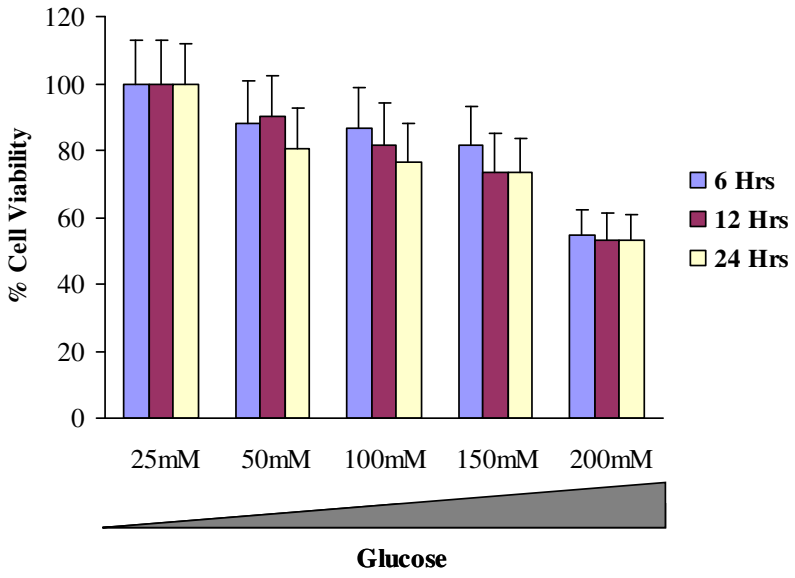


Figure 3. Cell Viability (MTT assay): RAW cells were treated with increasing concentration of glucose for 6, 12 and 24 hrs. At the end of each time point the cells were incubated in the presence of 1mg/ml of MTT dye and observed for the formation of formazan crystals after 2-3 hrs. The results are expressed as % cell viability as compared to control (100%). Data represent mean ± S.E. of three separate experiments (n =12).

3.4 Hyperglycemia Elevates Antioxidant Scavenging Enzymes: An important measure of oxidative stress in cells is the activation of scavenging enzymes involved in antioxidant defense mechanisms. Therefore the effects of hyperglycemia on activation of antioxidant enzymes Catalase, Cu-Zn superoxide dismutase [(Cu-Zn)-SOD] and Glutathione Reductase was examined using reverse transcription and PCR (RT-PCR) and proteomics. RT-PCR analysis of catalase gene expression in RAW macrophages exposed to high glucose showed an increase in catalase expression (**Fig 4A)**, 1.3-fold over NG controls. Treatment of cells with ascorbic acid (1 mM) and insulin (1 μM) even in the presence of high glucose reduced the catalase expression to 0.9-fold and 0.8-fold, respectively (**Fig 4A).**

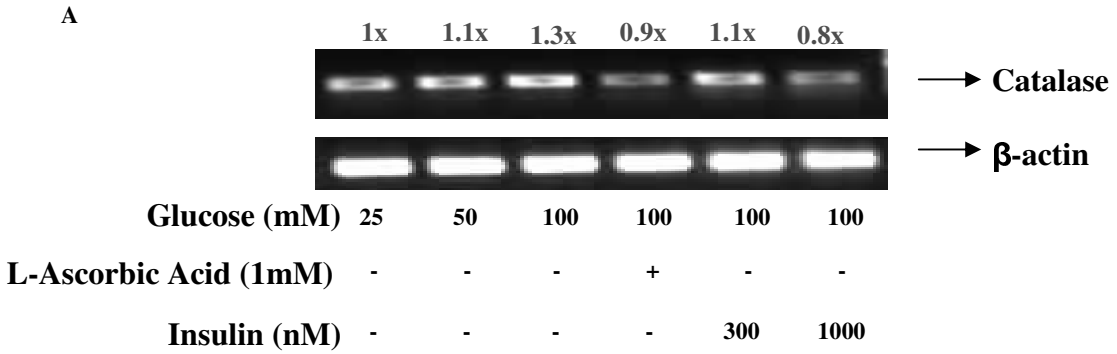
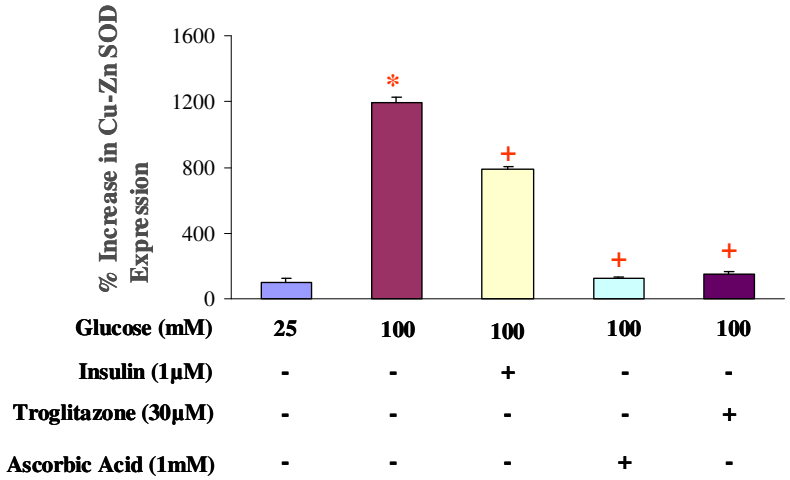


Figure 4. Elevated glucose upregualtes antioxidant enzyme levels in mouse macrophages: (A), The mRNA expression levels of catalase in macrophages exposed to high glucose with or without insulin and antioxidant ascorbic acid. The amount of catalase was normalized to the level of β-actin mRNA. All the data are expressed as fold relative to normal glucose (control).

B



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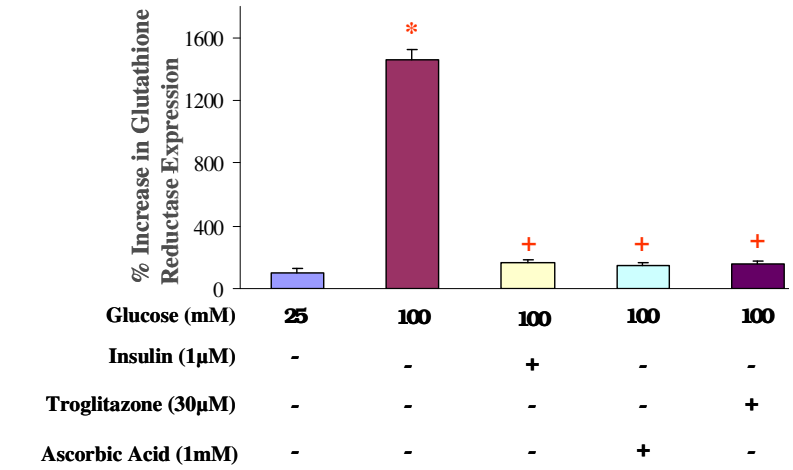


Figure 4. Elevated glucose upregualtes antioxidant enzyme levels in mouse macrophages: (B) & (C), Protein expression level of Cu-Zn SOD and Glutathione Reductase in macrophages exposed to high glucose with or without insulin, antidaibetic drug troglitazone and antioxidant ascorbic acid. Bars show the densitometry data of the spots from the 2D gel. The data is represented as percent increase as compared to control (25mm glucose). Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$). Plus (+) indicates significant difference over 100mM glucose (High Glucose) treatment alone ($p \leq 0.005$).The results are representative of three independent experiments and averaged.

The induction of (Cu-Zn)-SOD and glutathione Reductase was tested at the protein levels by proteomics approach in macrophages exposed to high glucose. Under hyperglycemic condition there was a significant increase in the level of (Cu-Zn)-SOD (**Fig 4B**) and glutathione reductase (**Fig 4C**). Treatment of cells with insulin (1 μM), ascorbic acid (1 mM) and troglitazone (10 μM) had reduced the expression of (Cu-Zn)-SOD (**Fig 4B**) and glutathione reductase protein levels (**Fig 4C**). These results indicate that hyperglycemia induces ROS generation, where the antioxidants and scavenging enzymes are induced neutralize the harmful effects of ROS and protect the cells.

3.5 Hyperglycemia Elevates Lipoxygenase Enzymes: The oxidative modification of low density lipoprotein (LDL) is an important event in the development of atherosclerosis. Macrophages are considered as a likely candidate to mediate in vivo LDL oxidation, because they are accumulated in the atherosclerosis lesions and are capable of in vitro LDL oxidation in culture medium free of metal ion additives (Brown and Goldstein, 1990; Chisolm et al., 1999; Parthasarathy et al., 1989). A number of evidences suggest that lipoxygenase enzymes contribute to LDL oxidation due to increased generation of ROS in activated macrophages (Goldstein et al., 1979; Kim et al., 1995; Mehrabian and Allayee, 2003; Spanbroek et al., 2003). Therefore the effects of hyperglycemia on activation of 5 lipoxygenase and 12 lipoxygenase enzymes were examined using proteomics. As shown in (**Fig 5A** (5-lipoxygenase) and **5B** (12-lipoxygenase)) incubation of macrophages in high glucose increased the

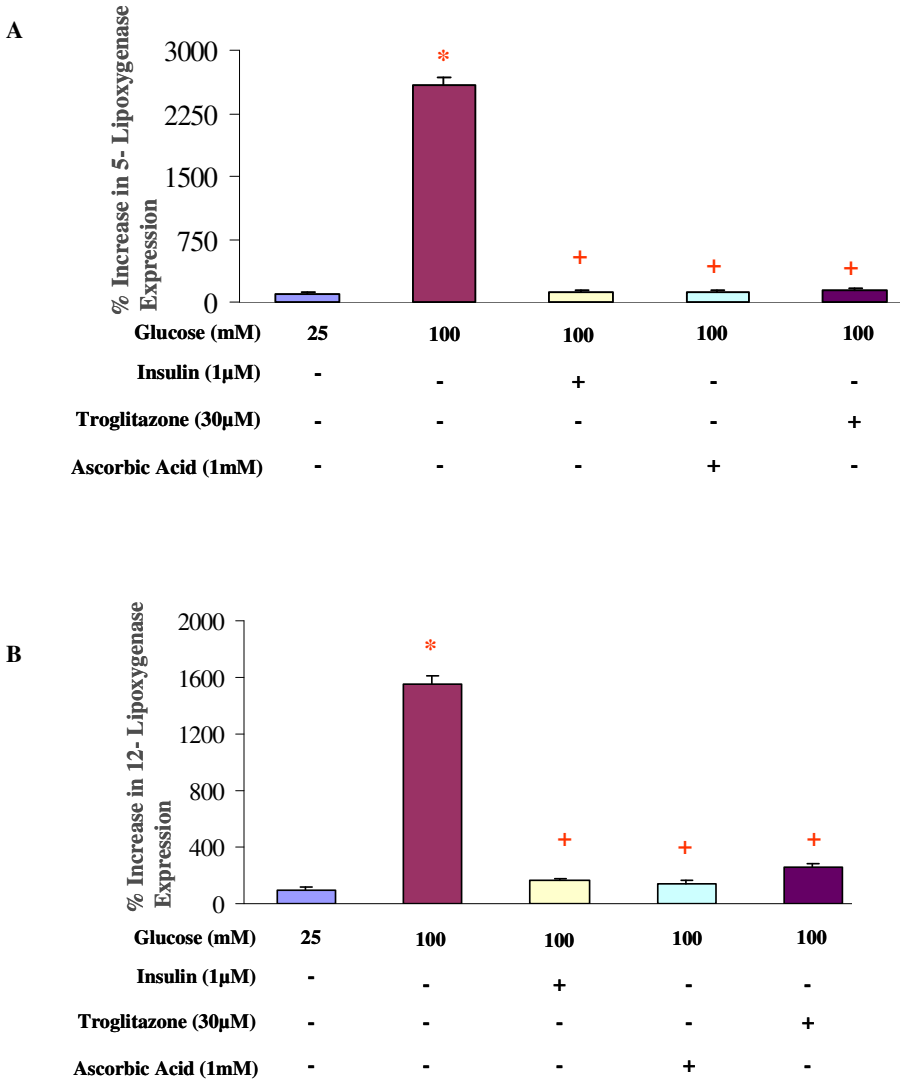


Figure 5. Elevated glucose upregualtes lipoxygenase enzyme levels in mouse macrophages. (A) & (B), Protein expression level of 5-lipoxygenase and 12-lipoxygenase in macrophages exposed to high glucose with or without insulin, antidaibetic drug troglitazone and antioxidant ascorbic acid. Bars show the densitometry data of the spots from the 2D gel. The data is represented as percent increase as compared to control (25mm glucose). Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$). Plus (+) indicates significant difference over 100mM glucose (High Glucose) treatment alone ($p \leq 0.005$). The results are representative of three independent experiments and averaged.

protein expression levels of lipoxygenase enzymes significantly as compared to macrophages incubated in normal glucose. In contrast, co-treatment of cells with insulin, ascorbic acid and troglitazone reduced the expression levels almost to the normal glucose levels. These results suggest that increased lipoxygenase enzyme activity observed in atherosclerotic lesion is partly mediated through hyperglycemia induced oxidative stress.

3.6 Hyperglycemia induced oxidative stress results in an increased inflammatory response: Activated macrophages have been implicated as key pathogenic regulators of the atherogenic process. The proinflammatory cytokines, TNFα, MMP9 and MCP-1 are released by activated macrophages and are present in the atherosclerotic lesion. TNFα is potent cytokine involved in inflammation and elevated levels of TNFα are seen in atherosclerotic plaque of diabetics. To evaluate the effect of hyperglycemia on the release of TNFα, RAW macrophages were exposed to high concentration of glucose. Hyperglycemia induced a significant increase in TNFα accumulation compared to the normoglycemic control (**Fig 6A**). However, when the cells were treated with various concentrations of insulin in presence of high glucose, TNFα induction had reduced significantly at 300 nM and 1 μM concentration of insulin (**Fig 6B).**

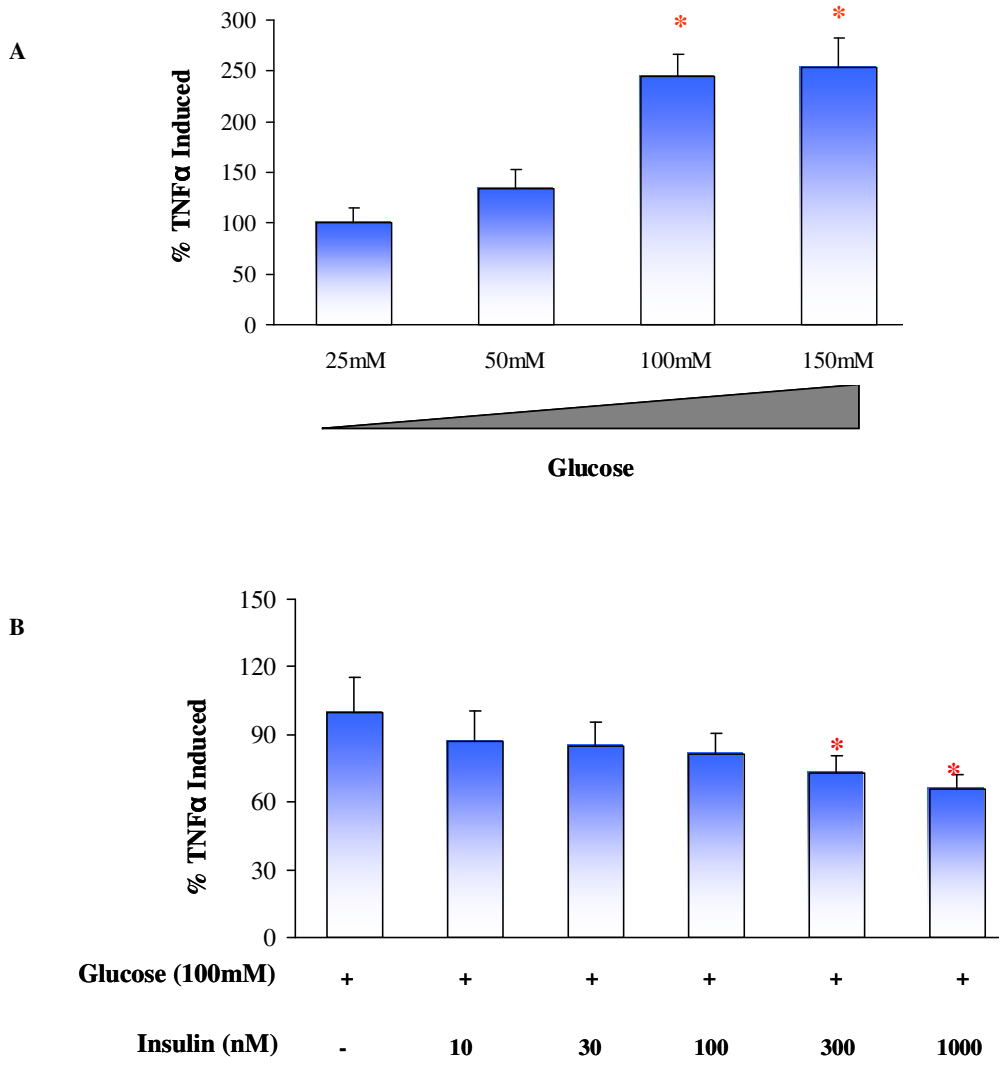


Figure 6. High glucose upregulates the pro inflammatory response in macrophages. **(A),** Macrophages were cultured in increasing of concentration glucose for 24 h. Medium was collected and TNFα was quantified using ELISA. **(B)**, Macrophages were cultured in high glucose with or without different concentrations of insulin for 24 h. Medium was collected and TNFα was quantified using ELISA. Data represents the mean ± S.E of three different experiments, each sample run in triplicate. Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$).

Next, to evaluate the possible induction of another two important cytokines MCP-1 and MMP-9 in diabetic condition, human cell migration, and derangement of MMP regulation is considered to be a critical factor in the development of vascular lesions. Macrophages were isolated from human blood (as described in experimental procedure) and incubated in high glucose medium for 24 hrs to evaluate the release of

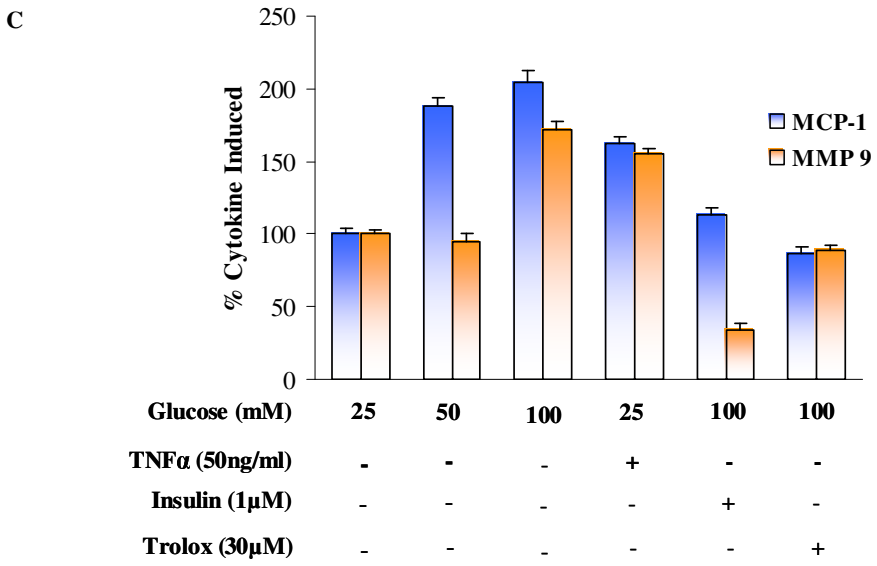


Figure 6. High glucose upregulates the pro inflammatory response in macrophages. **(C)**, Primary human macrophages were isolated and cultured as outlined under "Materials and Methods". MCP1 and MMP9 were quantified in the medium by ELISA. Each sample was run in triplicate.

cytokines in to the medium. High glucose induced a significant release of MCP-1 (**Fig 6C**, 100%) and MMP-9 (**Fig 6C**, 72%) into the media of human macrophage cells compared to normal glucose condition. Treatment of macrophages with insulin

(1 μM) and antioxidant trolox (30 μM) on the other hand reduced the level of MCP-1 and MMP-9 significantly (**Fig 6C**). This suggests that an increase in inflammatory response in hyperglycemic condition is mediated in part through elevated ROS levels in macrophages.

3.7 TNFα induces intracellular ROS production in RAW macrophages: It has been reported that TNFα is induced under hyperglycemic conditions in monocytes (Guha et al., 2000) and our earlier results show that exposing macrophages to high glucose increases ROS generation. Therefore the role of TNFα on the generation of ROS in macrophages was evaluated under normoglycemic conditions. As shown in (**Fig 7A)**, treatment of RAW macrophages with TNF-α (50 ng/ml) causes a rapid induction of intracellular ROS release. Interestingly, TNFα also induces secretion of MCP-1 and MMP-9 from macrophages under such normoglycemic condition (**Fig 6C**). However, when macrophages were simultaneously incubated with ascorbic acid (100 μM) and insulin (1μM) with TNFα there was a significant reduction in ROS release from TNFα stimulated cells **(Fig 7B)**. These results suggest that TNFα itself is a major inducer of ROS generation in macrophages, and an additional release of TNFα during hyperglycemic condition seems to further aggravate the condition.

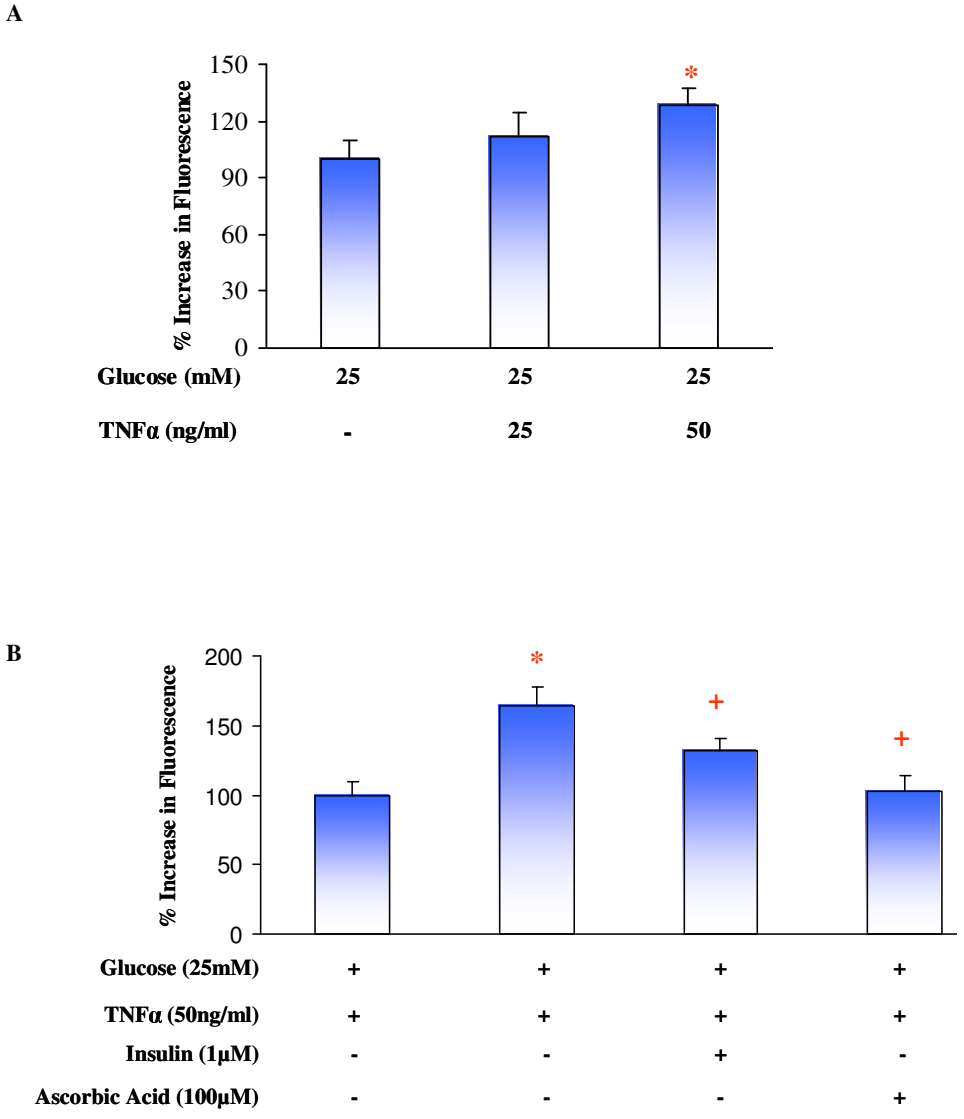


Figure 7. TNFα induces ROS generation in RAW 264.7 macrophages. (**A**), Macrophages cultured in normal glucose with increasing concentration of TNFα for 24 h. Intracellular ROS was determined as described under "Experimental procedure". Data are mean ± S.D, n = 12. Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$). **(B),** RAW macrophages were cultured in normal glucose containing 50ng/ml TNFα with or without insulin 1μM and ascorbic acid 100μM for 24 h. Intracellular ROS was determined. Data are mean ± S.D, n = 12. Asterisk (*) indicates significant difference over 25mM glucose (control) treatment alone ($p \leq 0.005$). Plus (+) indicates significant difference over 50ng/ml TNFα alone ($p \leq 0.005$).

3.8 Identification and regulation of FALDH in RAW macrophages: Hyperglycemia induced oxidative stress, via the production of ROS, results in oxidative chemical modification of lipids, DNA and proteins in various tissues. Such lipid peroxidation events cause damage to the tissues and thus need to be prevented. Normal cells protect themselves by producing detoxification enzymes, such as FALDH which is thought to be important for detoxification of both exogenous and endogenous aldehydes derived from lipid peroxidation of membrane phospholipids. Therefore FALDH modulation was investigated in hyperglycemic condition, since FALDH gene expression was shown to be regulated by insulin. Since macrophages are involved in development of atherosclerosis in diabetic patients, *in vitro* experiments were carried out in RAW cells to evaluate the modulation of FALDH under hyperglycemic condition. To first determine whether FALDH is expressed in RAW macrophages, cells were cultured in increasing concentration of glucose for 12hrs and 24hrs, total RNA was extracted and the FALDH expression was examined by northern blot. Basal expression of FALDH was detected in RAW macrophages, which decreased in a dose dependent fashion upon exposure to high glucose (Fig **8A**). There was a reduction in FALDH expression by 25% and 38% at 12 hrs and 24 hrs respectively when cells were incubated with 100 mM glucose compared to the basal level (**Fig 8A**). Similar results were also observed when cells were treated with increasing concentration of H_2O_2 which generates ROS **(Fig 8B).**

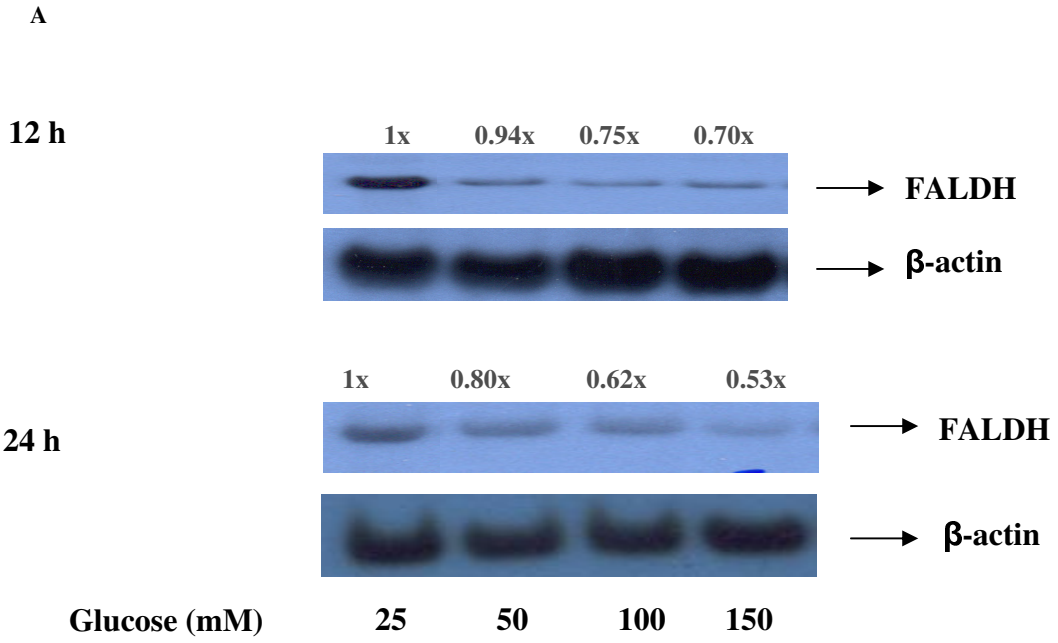


Figure 8. FALDH gene expression in Mouse Macrophages. (A) Macrophages were treated with increasing concentration of glucose for 12h and 24 h as indicated. After each time point, total RNA was extracted and expression of FALDH mRNA was analyzed by Northern blot using a ^{32}P-labeled FALDH cDNA probe. The amount of FALDH expression was normalized to the level of β-actin mRNA. All the data are expressed as fold relative to normal glucose (control). The results shown are representative and average of three independent experiments (n = 3).

In earlier report (Demozay et al., 2004), experiments were carried out in rat liver and isolated rat adipocytes where it was shown that insulin induces FALDH expression. Therefore, it was tested whether similar regulation by insulin was there in macrophages.The expression of FALDH was examined in RAW macrophages, where cells grown in normoglycemic condition were treated with increasing concentrations of insulin. The northern blot analysis showed that FALDH expression

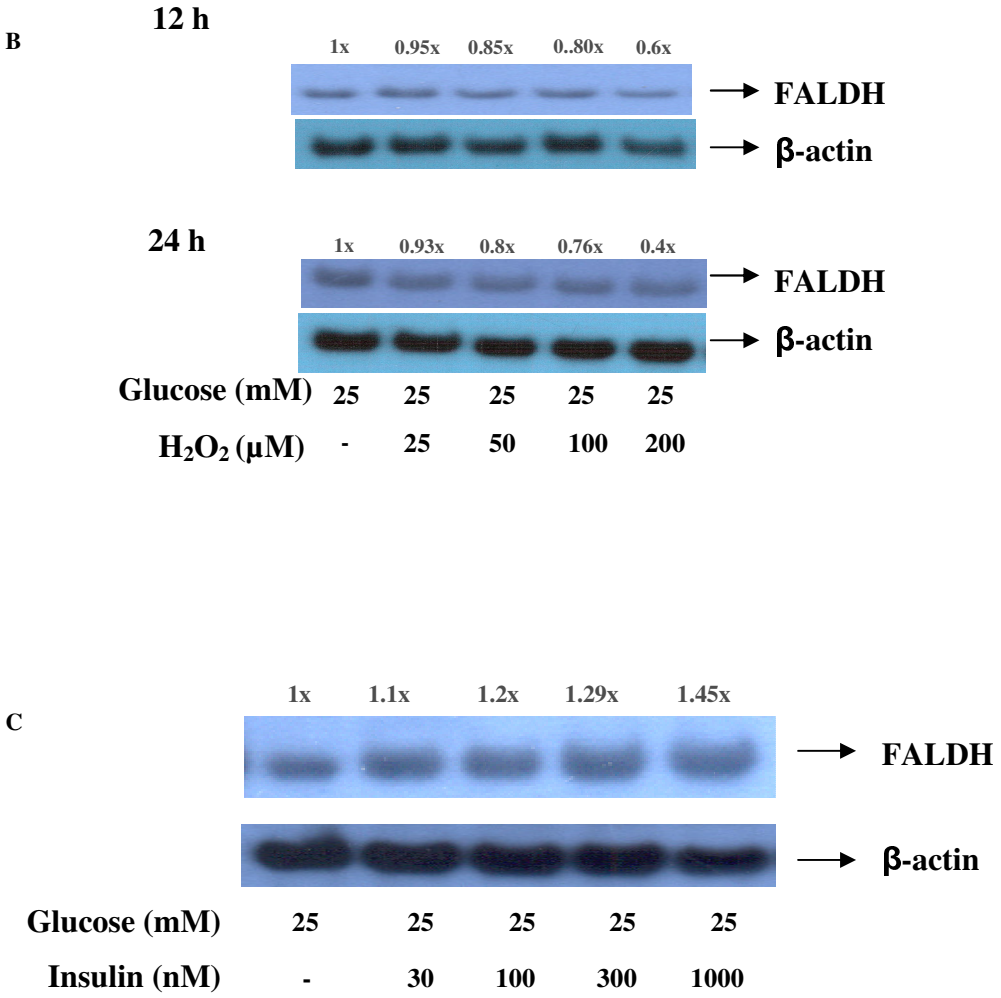


Figure 8. FALDH gene expression in Mouse Macrophages. (B) Macrophages were treated with increasing concentration of H_2O_2 for 12h and 24 h as indicated. After each time point, total RNA was extracted and expression of FALDH mRNA was analyzed by Northern blot using a ^{32}P-labeled FALDH cDNA probe. (**C**), To confirm insulin stimulation of FALDH, cells were treated with increasing concentration of insulin for 24 h. Total RNA was extracted and expression of FALDH mRNA was analyzed by Northern blot. The amount of FALDH expression was normalized to the level of β-actin mRNA. All the data are expressed as fold relative to normal glucose (control). The results shown are representative and average of three independent experiments (n = 3).

was elevated with increasing insulin concentration (**Fig 8C**), validating the approach. These results which show that FALDH expression is regulated by insulin and reduced in hyperglycemic conditions, confirm that oxidative stress leads to down regulation of insulin mediated PI3 kinase regulated genes and thus may participate in the pathogenesis of type 2 diabetes and insulin resistance.

3.9 Effect of TNFα on FALDH mRNA expression: In an earlier experiment it was shown that treating macrophages with TNFα under normoglycemic condition results in ROS generation. To evaluate whether TNFα modulates FALDH expression, via the production of ROS, RAW cells were cultured in normoglycemic condition and treated with increasing concentration of TNFα for 24hrs. Total RNA was extracted from treated cells and the FALDH expression was examined by RT-PCR.

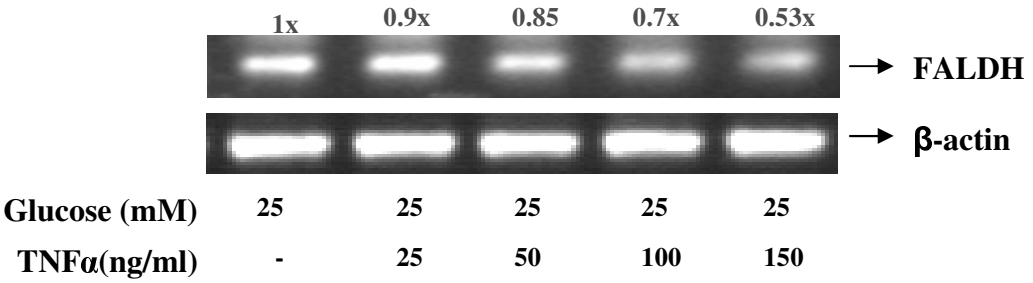


Figure 9. TNFα modulates FALDH expression levels. RAW macrophages were treated with various concentrations of human TNFα recombinant protein. After 24 hrs total RNA was extracted and analyzed by RT-PCR using FALDH primers. mRNA expression was normalized using β-actin RNA levels. The results are expressed as fold relative to normal glucose (control).

As shown in (**Fig 9)**, treatment of RAW macrophages with TNF-α caused a reduction in FALDH expression in a dose dependent manner of 15% at 50 ng/ml and 50% at 150 ng/ml of TNF-α. This data suggest that the downregulation of FALDH may be partly mediated by increased secretion of TNFα caused by hyperglycemia induced oxidative stress.

3.10 Insulin and antioxidants inhibit down regulation of FALDH mRNA expression in oxidative stress condition: Previous experiments have shown that FALDH expression is downregulated due to hyperglycemia induced oxidative stress and antioxidants can quench the ROS that is generated due to hyperglycemia. Thus wanted to investigate whether the hyperglycemia induced FALDH downregulation can be inhibited by insulin and antioxidants. RAW macrophages were simultaneously treated with high glucose and insulin and FALDH gene expression was examined by Northern blot. As expected FALDH expression was downregulated in presence of high glucose (**Fig 10A**). However, co-treatment with insulin prevented the downregulation of FALDH caused by hyperglycemia and FALDH expression was found to be upregulated in a dose dependent manner (**Fig 10A**). Similar results were observed with macrophages subjected to co-treatment with normal or high glucose and antioxidant (ascorbic acid) where FALDH expression levels were normalized to the basal level upon ascorbic acid treatment (**Fig 10B**). To summarize, the data confirms that hyperglycemic condition leads to a downregulation of FALDH

level due to oxidative stress, which can be prevented by treatment with antioxidant or insulin.

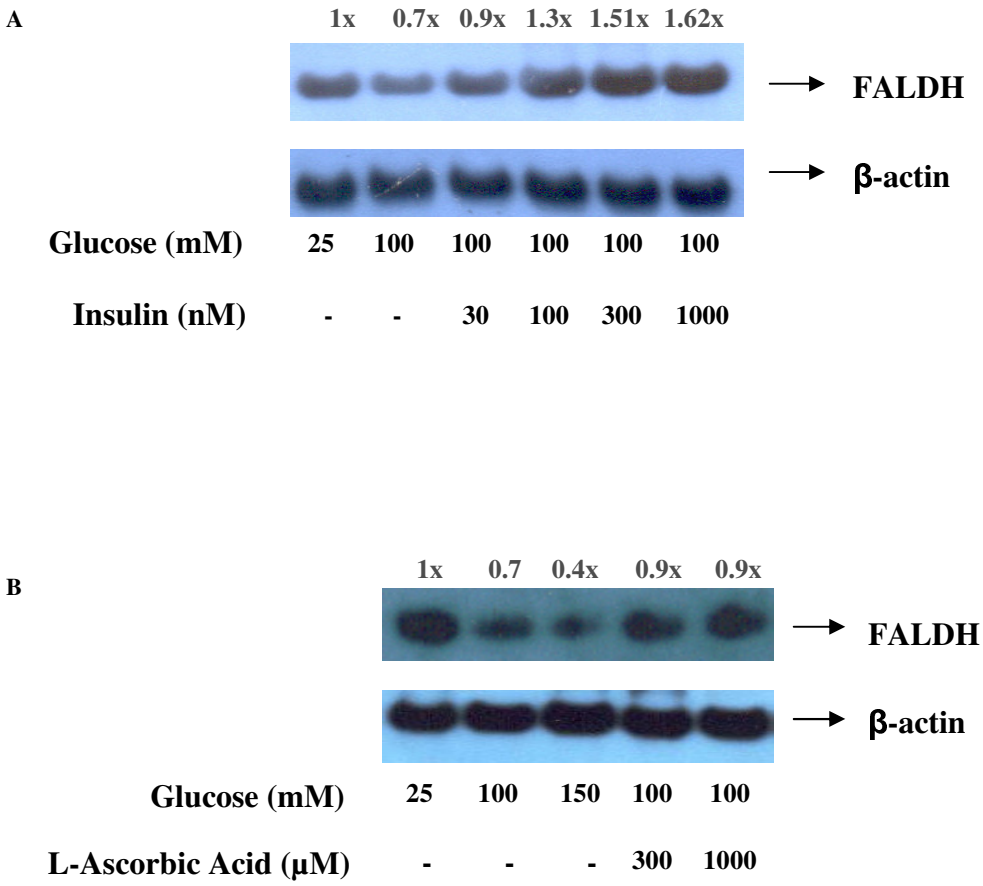


Figure 10. Reversal of FALDH gene expression by insulin and ascorbic acid under high glucose condition. (A) & (B), Macrophages were cultured in 100mM glucose (high glucose) with or without insulin and antioxidant ascorbic acid for 24 h. Total RNA was extracted and expression of FALDH mRNA was analyzed by Northern blot and normalized to β-actin. Similar results were obtained in three separate experiments and averaged.

3.11 Regulation of FALDH gene expression in STZ diabetic mice: To verify the role of hyperglycemia induced oxidative stress in the modulation of FALDH gene expression *in vivo*, BALBc mice were treated for 30 days with or without streptozotocin (100mg/kg), a drug that specifically destroys beta cells of the endocrine pancreas leading to increased glucose level, type 1 diabetes. Additionally, another group of animals were first treated with streptozotocin (STZ) for 15 days to induce hyperglycemia and then they were treated with both STZ and ascorbic acid (800mg/L in drinking water, STZ+AA) for another 15 days. Untreated controls, STZ treated and STZ+AA treated rats were euthanized after 30 days by injection, aorta and liver were collected from each animal. The whole aorta was used for RNA extraction since it was very difficult to isolate macrophages from them and moreover the development of atherosclerosis occurs in the aorta. Total RNA isolated from aorta was analyzed for FALDH expression by RT-PCR. As shown in (**Fig 11A**), basal FALDH expression was reduced by ~45% in STZ treated rats compared with control rats. In other group of STZ+AA treated animals, ascorbic acid protected STZ-induced FALDH downregulation (**Fig 11A).** To further confirm the role of hyperglycemia induced ROS in the downregulation of FALDH, the expression of a scavenging enzyme, catalase was checked in the aorta of the above three groups. RT-PCR analysis clearly showed that there is a significant increase in catalase gene expression in the aorta from STZ rats compared to control rats (**Fig 11B**). When mice were treated with ascorbic acid (STZ+AA), the increase in catalase expression is normalized to the basal level (**Fig 11B**), which indicates that ascorbic acid protects

the tissue from hyperglycemia induced oxidative stress. This data suggest that FALDH expression is reduced in the aorta of mice with STZ-induced type 1 diabetes and antioxidants can protect this effect *in vivo* to some extent.

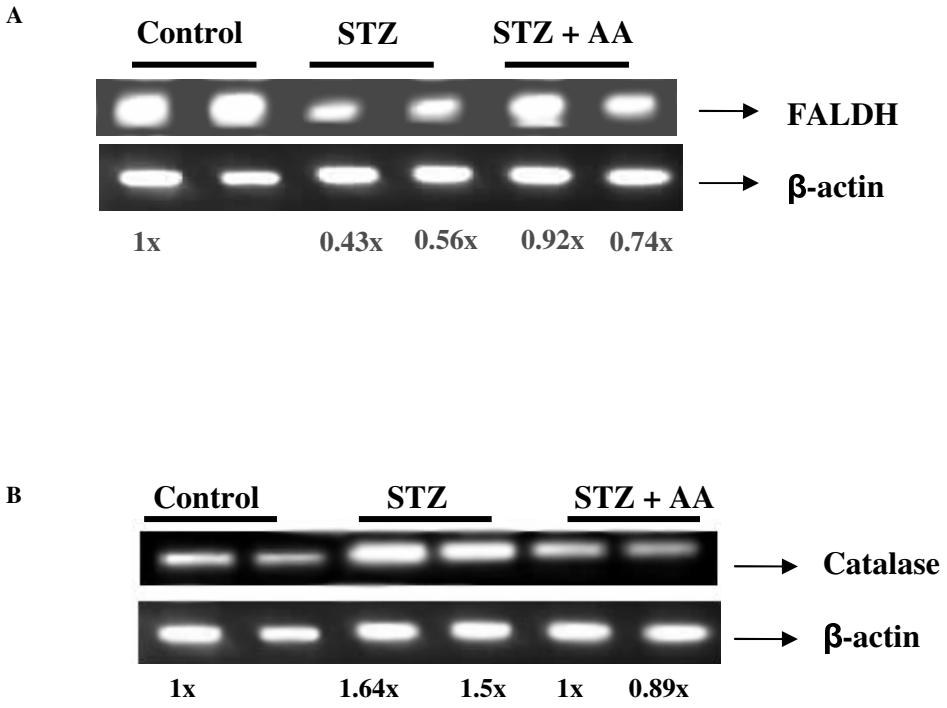


Figure 11. FALDH gene expression in aorta of type 1 diabetic mice (STZ). (A) & (B), BALBc mice were fasted overnight and then injected intraperitoneally with streptozotocin (100mg/kg) or with citrate buffer. After 15 days mice were divided into two groups (n = 5) based on plasma glucose levels. One group served as STZ control and other group was treated with sscorbic acid (800mg/kg) in water and continued for another 15 days. Mice were euthanized and total RNA was extracted from the aorta. FALDH and Catalase gene expression was analyzed by RT-PCR using FALDH and catalase primers. mRNA expression was normalized using β-actin mRNA levels. The results are expressed as fold relative to normal BALBc mice which were not treated with streptozotocin.

3.12 Expression of FLADH gene in Insulin-resistant diabetic mice: Insulin resistant condition found in type 2 diabetes is characterized by attenuation of the PI3K signaling cascade due to defective insulin signaling which results in downregulation of the genes that are regulated by insulin. Thus, FALDH gene expression was studied to check whether it is affected by insulin resistance in a type 2 diabetes animal model, *db/db* mice. 12-week old *db/db* mice and control mice C57BL/6 were euthanized and aorta was collected. RNA was extracted from the aorta and FALDH gene expression was analyzed by RT-PCR. In control mice, significant FALDH mRNA expression was observed in the aorta, which was found to be severely down regulated (~70%) in *db/db* mice (**Fig 12**). This data suggest that FALDH expression is downregulated in insulin resistance type 2 diabetic db/db mice.

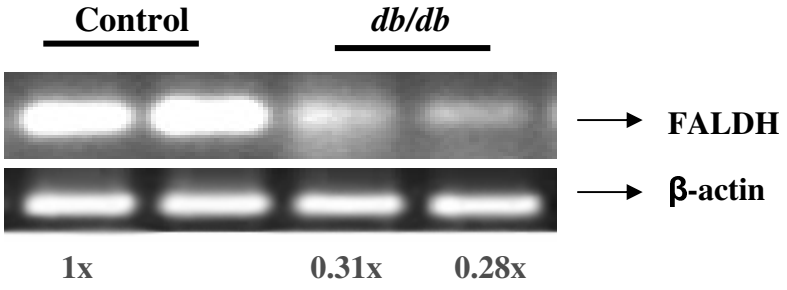


Figure 12. FALDH gene expression in aorta of insulin resistant mice (*db/db*). *db/db* C57BL/6J and lean controls (n=2) were sacrificed at 12 weeks of age and total RNA was extracted from the aorta. FALDH expression was analyzed by RT-PCR and was normalized using β- actin mRNA levels.

3.13 FALDH Enzyme Activity in various diseased models: To investigate whether the downregulation of FALDH observed at mRNA level is reflected at the enzyme activity, microsomes were isolated from the liver and FALDH enzyme activity was checked in various diabetic and obese animal models. FALDH was assayed spectrophotometrically by monitoring the octanal dependent production of NADH. Using liver microsomes, octanal dependent NADH production was monitored over time period of 5 min.

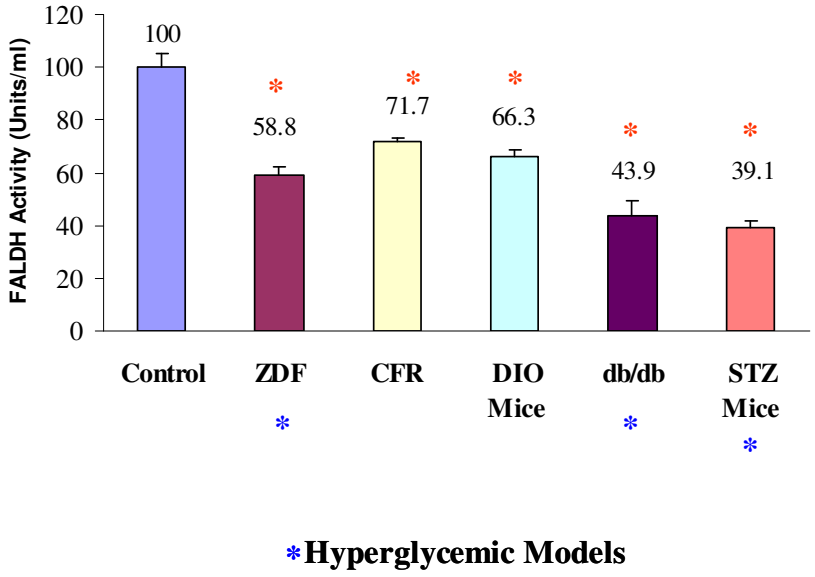


Figure 13. FALDH enzyme activity in the liver of diseased animal models. Diabetes, dyslipidemia, and obese rats and mice with respective controls (n=3) were sacrificed and liver was immediately collected. Microsomes were isolated from the liver as described in "Experimental procedure". FALDH enzyme activity was analyzed spectrophotometrically by monitoring the octanal dependent production of NADH. Each sample was run in triplicate and results were averaged. Asterisk (*) indicates significant difference as compared to control alone ($p \leq 0.005$).

As shown in (**Fig 13)** the FALDH enzyme activity was reduced by 60% in various diabetic animal models having high glucose levels as compared to the controls which were normoglycemic. The enzyme activity was then checked in high fat diet dyslipidemia rat model and diet induced obese mouse model, a more representative of metabolic syndrome with mild insulin resistance and hyperglycemia. In both dyslipidemia and diet induced obese model there was only a 30% reduction in FALDH enzyme activity as compared to the hyperglycemic condition. This data clearly indicate that the downregulation of FALDH is mediated through hyperglycemia induced oxidative stress.

3.14 Confirmation of FALDH mRNA data with the protein data- Data from 2D gel electrophoresis and MALDI-TOF: 2-DE in conjunction with quantitative image analysis was used to investigate modulation in the protein expressing profile of FALDH in macrophages. Three gels per sample were processed simultaneously and analyzed with PDQuest – 2D software. (**Fig 14A)** shows one of the 2-D silver stained gel (pH 3-10) which was used for comparative and semi quantitative spot analysis. More than 300 proteins showed significant protein modulation as compared to control and FALDH spot was identified based on pI, molecular weight and mass fingerprint. Protein spots from the 2D gel were subjected to trypsin digestion and identified by MALDI-TOF mass spectrometer. Of the many differentially expressed spots, FALDH spot (shown in **Fig 14A**) was identified with its mass fingerprint and comparison with the mass obtained from the Swiss-port database. The data seen in

(Fig 14B) shows that FALDH protein gets significantly downregulated under high glucose condition and co treatment of hyperglycemic condition with insulin, ascorbic acid and troglitazone (antidiabetic drug) prevented the down regulation of FALDH.

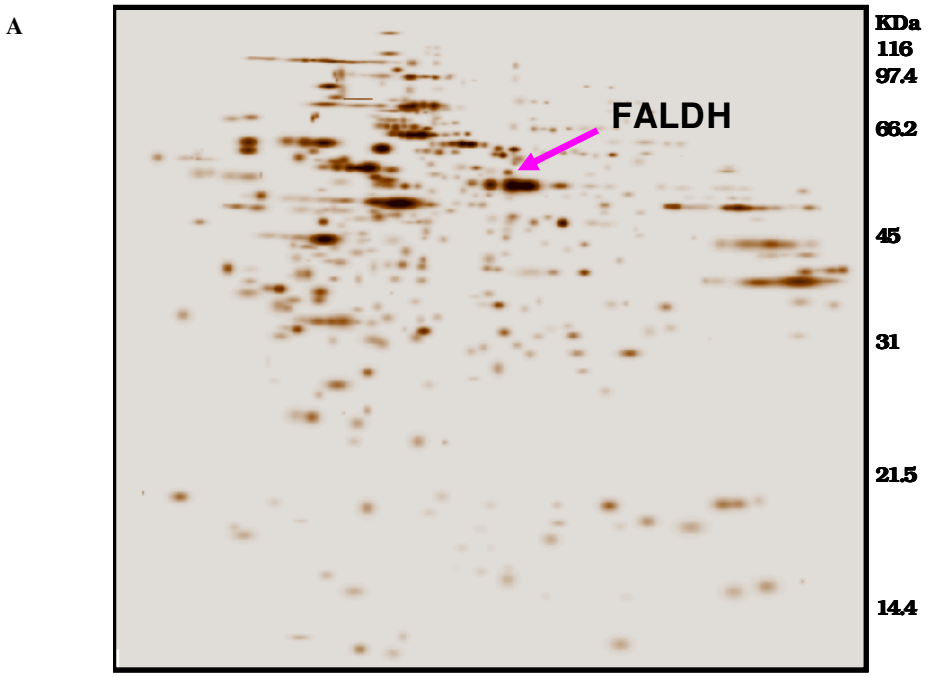


Figure 14. FALDH protein expression in 2Dgel and MALDI-TOF: (A), Macrophages were cultured in insulin, high glucose with or without insulin, ascorbic acid and troglitazone for 24 h. Protein was extracted as described in "Experimental and Methods" and separated on 2D gel electrophoresis. FALDH protein spot was identified by mass finger print based on pI and molecular weight.

To summarize, the results observed at the protein and at enzyme activity clearly indicate that hyperglycemic condition leads to a downregulation of FALDH due to oxidative stress which is not only observed at the transcriptional level but even

modulated at the translational level. Treatment with ascorbic acid (antioxidant), insulin and troglitazone (antidiabetic drug) prevents the down regulation of FALDH by quenching ROS. This data confirms that there is a perfect correlation between modulation of FALDH mRNA expression and FALDH protein function.

B

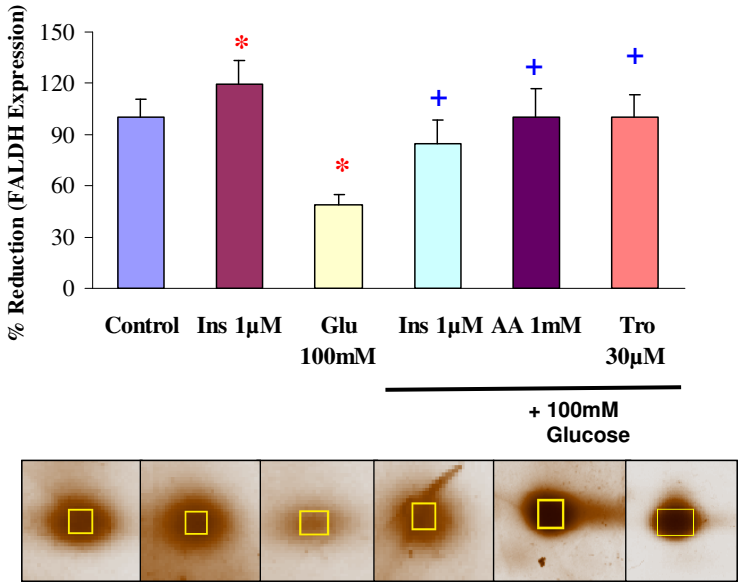


Figure 14. FALDH protein expression in 2Dgel and MALDI-TOF: (B), Protein spots from the 2D gel were subjected to trypsin digestion, quantified by densitometry and normalized to total protein levels. The data are represented as percent increase as compared to control (25mm glucose). Asterisk (*) indicates significant difference over 25mM glucose (control) alone ($p \leq 0.005$). Plus (+) indicates significant difference over 100mM glucose (High Glucose) treatment alone ($p \leq 0.005$).The results are representative of three independent experiments and averaged.

It is now well established that hyperglycemia is a major risk factor in the pathogenesis of diabetic complications and patients with Type 2 diabetes mellitus (T2DM) are associated with an increased risk of premature atherosclerosis (Esposito et al., 2002). Hyperglycemia induces a large number of alterations in vascular tissue that potentially promote accelerated atherosclerosis. One of the major mechanisms through which hyperglycemia causes cardiovascular problem is oxidative stress via the production of ROS resulting in increased production of superoxide radicals (Brownlee et al., 2001, Koya et al., 1998, Nishikawa et al., 2000, Du et al., 2000, Evans et al., 2002). Although these effects were earlier observed in other cell types such as endothelial cells, adipocytes and vascular smooth muscle cells, very little is known about the molecular mechanism of how diabetes induced ROS affect macrophages, the cells that play a major role in development of atherosclerosis. Therefore in the present study the effect of hyperglycemia induced ROS in macrophages was examined, since macrophages are the cells that generate ROS upon stress, release immune mediators and uptake oxidized-LDL, which collectively contribute to atherosclerosis. In this study it is demonstrated for the first time that hyperglycemia induces oxidative stress in macrophage cells by stimulating production of ROS. The hyperglycemia induced oxidative stress was measured by detecting the ROS generation using DCF-DA dye, which could be quenched by various antioxidants. The generation of ROS was confirmed by measuring the modulation in expression of key antioxidant scavenging enzymes, Catalase, Cu-Zn superoxide dismutase [(Cu-Zn)-SOD] and Glutathione Reductase by RT-PCR and

proteomics analyses. Subsequently, it was shown that the culturing of macrophages in high glucose concentration caused a dramatic increase in the release of inflammatory cytokine, chemokine and related molecules. A significant two fold increase was observed in the expression of cytokine TNFα, chemokine MCP-1, and metalloproteinase MMP-9. In the present study modulation of a novel gene in macrophages by oxidative stress due to hyperglycemic conditions both *in vitro* and *in vivo* is being reported.

Mouse macrophages RAW264.7 cells were used in the study, since these cells have been routinely used for studying macrophage function. Differentiated human THP-1 macrophages were avoided to study hyperglycemia induced oxidative stress, since differentiation of THP-1 monocytes to macrophages with PMA by itself resulted in generation of ROS. Previous studies have shown that PMA induced ROS generation is mediated through activation of PKC (Hsu et al., 2000). Additionally, initial experiments were carried out with human vascular smooth muscle cells (hVSMC), which showed results similar to that with RAW264.7 cells (data not shown). However, further experiments were not carried out with hVSMC since they are primary cell line and difficult to grow in large quantity.

Hyperglycemia in the blood stream causes oxidative stress to circulating monocytes due to increased production of mitochondrial ROS (Brownlee et al., 2001), nonenzymatic glycation of proteins and generation of peroxide species by glucose autooxidation (Evans et al., 2002). ROS can stimulate oxidation of LDL, and ox-LDL, which is not recognized by the LDL-receptor, can be taken up by

scavenger receptors in macrophages leading to foam cell formation and atherosclerotic plaques (Gao et al., 1996, Ceolotto et al., 1999, Yim et al., 1995, Hofmann et al., 1999). In this study it has been demonstrated that culturing of macrophage cells in high glucose induced oxidative stress by stimulating ROS generation. This was shown using DCF-DA dye, where cells exhibit high fluorescence when they are subjected to oxidative stress, which was ameliorated by co-treatment with insulin and various antioxidants. The data demonstrated the close link of nutrient excess in the form of hyperglycemia, and the concomitant increase in ROS in macrophages as compared to cells grown in normal glucose. These results are in accordance with previous data shown in other cell lines (Nishikawa et al., 2000, Lin et al., 2005).

Excessive levels of ROS lead to damage of proteins, lipids and DNA (Evans et al., 2002). Thus, endogenous antioxidant systems exist in cells that neutralize the ROS generated, and these systems are critical to maintaining proper cellular function. In the absence of such an endogenous antioxidant network, the system becomes overwhelmed (redox imbalance), leading to the activation of stress-sensitive intracellular signaling pathways. A major cellular antioxidant is reduced glutathione (GSH), which is regenerated efficiently by glutathione reductase (Young et al., 2001; Gibson et al., 1985). Superoxide dismutase is a naturally occurring enzyme that protects the body against active oxygen radicals by scavenging excess superoxides, thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives (Young, et al., 2001, Morten et al., 2006).

Superoxide dismutases (SODs) catalyze the dismutation of two superoxide anion radicals to molecular oxygen and hydrogen peroxide (H_2O_2). The hydrogen peroxide must then be removed by catalase. Catalase is an antioxidant enzyme that is predominantly located in peroxisomes, and it functions by abrogating the cytotoxic effects of hydrogen peroxide by catalyzing the conversion of hydrogen peroxide to water and oxygen (Young et al., 2001, Morten et al., 2006). In the present study, an upregulation in catalase gene expression was observed in hyperglycemic condition. Similar effects were also observed for (Cu-Zn) SOD and glutathione reductase at protein level (>1000 fold increase), when macrophages treated under hyperglycemic conditions were analyzed by proteomics approach. This increase in superoxide radical was diminished when cells were treated with antioxidants, such as troglitazone, ascorbic acid or insulin. The data shows that cells upregulate the antioxidant defense mechanism to protect from the cytotoxic effects of superoxide radicals that are generated during chronic hyperglycemic conditions such as type 2 diabetes.

Hyperglycemia causes a change in the redox status in the cells, leading to the activation of key inflammatory proteins by modulating their gene expression (Guha et al., 2000, Yamakawa et al., 1999). Previous studies have shown that in macrophages ROS increases the production of various cytokines like TNF-α and chemokines MCP-1 by activation of NF-κβ pathway (Reape et al., 1999, Peters et al., 2001). Activated macrophages have been implicated as key pathogenic regulators

of the atherogenic process. The proinflammatory cytokines, TNFα, MCP-1 and MMP-9 are released by activated macrophages and are present in the atherosclerotic lesion. TNFα is involved in inflammation and elevated level of TNFα is known to cause insulin resistance through the activation of different serine/threonine kinases, such as MAPK (JNK, ERK, and p38) and IKK (Fujishiro et al., 2003). Interesting it has also been shown that TNFα stimulates ROS generation, both *in vitro* and *in vivo*, in various cell lines and tissues such as macrophages, rat liver hepatocytes and pulmonary tissues (Mukhopadhyay et al., 2006, Corda et al., 2001). Monocyte chemoattractant protein 1(MCP-1) is a potent chemoattractant and is involved in infiltration of macrophages, inflammation cytokines, adhesion molecule expression and superoxide production (Furukawa et al., 2004). It has recently been shown that ROS and ox-LDL (byproducts of lipid peroxidation) found in diabetic patients are potent inducer of MCP-1 in endothelial cells, vascular smooth muscle cells, and monocytes (Cushing et al., 1990, Takahara et al., 1997). MMP-9, a matrix metalloproteinase, is involved in monocyte invasion and vascular smooth muscle cell migration; and derangement of MMP regulation is considered to be critical for development of atherosclerosis (Uemura et al., 2001). It is known that MMPs are activated by ROS and their expression is upregulated during oxidative stress. In diabetic patients where the prevalence of acute coronary syndrome is higher compared to the non-diabetic patients, MMP9 expression and activity was found to be preferentially higher. It was demonstrated that hyperglycemia induced oxidative

stress appeared to play a primary role in the activation of MMP-9 in endothelial cells of diabetic patients (Uemura et al., 2001). Similar results were observed in our experiments using mouse macrophage RAW264.7 cells, where treatment with high glucose concentration led to a significant induction of TNF-α, MCP-1 and MMP9 (Fig 3). To confirm whether increased expression of various inflammatory cytokine and chemokines in macrophages is mediated by ROS generated due to oxidative stress, the cells were cultured in hyperglycemic condition and co-treated with various known antioxidants like trolox, ascorbic acid, and insulin. Our results showed that when the macrophage cells were co-treated with the antioxidants there was a significant reduction in inflammatory cytokine and chemokine levels. These data suggested that induction in inflammatory signal generated due to oxidative stress were mediated through ROS generation in response to hyperglycemia.

Although various signaling pathways are known to be involved in the development of CVD in diabetic patients, the detailed molecular mechanism of these processes are not fully understood. To better understand and identify the role of various proteins that might be modulated by hyperglycemia induced oxidative stress, effect of oxidative stress on global protein modulation was studied in macrophage cells using proteomic approach. In macrophages various proteins were identified using this approach that were significantly modulated and are known to be upregulated by ROS. Among these, 5-lipoxygenase, 12-lipoxygenase, (Cu-Zn) SOD, Glutathione reductase were identified as key proteins that were modulated in

hyperglycemic condition. The significance and modulation of (Cu-Zn) SOD and Glutathione reductase by hyperglycemia induced ROS was discussed earlier.

Next the other two proteins that are significantly modulated and play an important role in development of atherosclerosis in diabetic patients are lipoxygenases. Lipoxygenases are a family of non-heme iron containing dioxygenases that incorporate one molecular oxygen into unsaturated fatty acids giving rise to their hydroperoxy derivatives. Increased expressions of lipoxygenases have been reported in the atherosclerotic plaque of diabetic patients. These enzymes have been classified as 5, 8, 12 and 15 lipoxygenases in mammalian tissues, named according to the number of carbon atom on which oxygenation of arachidonic acid takes place (Xuet al., 2001, Cathcart et al., 2000). 12-lipoxygenase plays an important role in the development of atherosclerosis in diabetic patients by increasing the cellular eicosanoid production. The eicosanoid 12(S)-hydroxyeicosatetraenoic acid (12-S-HETE) and 15 (S) hydroxyeicosatetraenoic (15-S-HETE) acids are produced by incorporating molecular oxygen to arachidonic acid under hyperglycemic conditions by the macrophages. The eicosanoid production facilitates the binding of the macrophages to the aortic endothelial cells and vascular muscle cells (Brown et al., 1988, Natarajan et al., 1993, Kim et al., 1994, Patricia et al., 1999). In addition to macrophage attachment, 12-Lox plays an important role in the oxidation of LDL by increasing the production of ROS and has been implicated in atherogenesis (Parthasarathy et al., 1989, Belkner et al., 2005, Cyrus et al., 2001). 5- lipoxygenase (5-Lox) is involved in the biosynthesis of pro-inflammatory

leukotrienes during the conversion of arachidonic acid to 5(S)hydroperoxy-6,8,11,14-eicosatetraenoate (5-HPETE) and has been implicated in the development of atherosclerosis (Funk et al., 2001, Spanbroek et al., 2003, Mehrabian et al, 2002). Increased 5-Lox enzyme activity in the monocyte/macrophages is found to be responsible for increased expression of adhesion molecules on the endothelial cells and firm binding of monocytes to adhesion molecules and transmigration into the subendothelial space. In the present study an upregulation of 5- and 12-lipoxygenases were observed in macrophages under hyperglycemic conditions compared to the control, which were normalized when the cells were treated with insulin ascorbic acid and troglitazone indicating the role of hyperglycemia induced oxidative stress in the upregulation of these enzymes. This data, together with the observation seen in other models indicate that hyperglycemia upregulates the lipoxygenases which play a role in the acceleration of atherosclerosis in diabetic patients by leading to oxidation of LDL.

In this study a novel protein fatty aldehyde dehydrogenase (FALDH) was identified, which was significantly modulated in macrophages under hyperglycemic condition. Macrophages are the cells that populate arteries, are activated by oxidative stress, uptake ox-LDL and contribute towards atherosclerosis. According to existing literature the expression and the role of FALDH in macrophage cells is not reported as of yet. The major function of fatty aldehyde dehydrogenase (FALDH) is to detoxify the exogenous and endogenous aldehydes resulting from lipid peroxidation (Traverso et al., 2002, Yoshida et al., 1998) and protect cells against harmful effects

of aldehydes (Baynes et al., 1991, Rosen et al., 2001). Previously Demozay et al., have shown, that FALDH is regulated by insulin *in vitro* and its expression is downregulated in insulin resistant type 2 and hypoinsulinemic type 1 diabetic condition *in vivo (*Demozay et al., 2004*)*. Although this study has shown that FALDH is downregulated in liver and adipose tissues in diabetic animal models (Demozay et al., 2004), the exact mechanism that results in the downregulation of FALDH is not clearly elucidated. Additionally, the authors have suggested that FALDH plays a role in protecting cells against lipid peroxidation during oxidative stress. Therefore the effect of hyperglycemia on the regulation and function of FALDH in macrophages was studied due to the following reasons. Some of the harmful effects of aldehyde accumulation are stiffening of arterial wall (Aronson et al, 2003), induce stress signaling pathways in macrophages and endothelial cells (Akhand et al., 2001, Chang et al., 2005) increase the generation ROS and cytokine production via NFκB activation (Lieuw-a-Fa et al., 2006), increases the expression of adhesion molecules (Fan et al., 2003), inhibit ABCA1 dependent reverse cholesterol transport, facilitate foam cell formation in macrophages (Passarelli et al., 2005) and lead to the development of atherosclerosis. Increased formation and accumulation of various protein-aldehydes adducts and weakened detoxification system was found in the aorta and heart during the course of diabetes (Phillips et al., 1993 McLellan etal., 1994, Oya et al., 1999, Randell et al., 2005). The information from various finding clearly indicates the harmful cardiovascular effects of aldehydes which are formed as by products of lipid peroxidation. To neutralize the

harmful effects of aldehydes the cells have various aldehydes dehydrogenase detoxifying enzymes (Vasiliou et al., 2000) like FALDH.

Activation of PI3K by insulin leads to the stimulating of numerous PI3K regulated genes which control lipid and glucose homeostasis, such as Akt, p38 MAPK, FAS, and FALDH. To show that FALDH is expressed in RAW macrophages and regulated by insulin, RAW cells were treated with increasing concentration of insulin and examined the expression of FALDH by northern blot. The northern blot data showed for the first time that FALDH is expressed in macrophages and is induced by insulin similar to what was observed with liver and adipose tissues (Demozay et al., 2004). This data suggests that insulin enhances the expression of FALDH and this might be one of the pathway through which insulin shows anti-athero protective properties in normal people. As it is not known how hyperglycemic conditions observed in type 1 and type 2 diabetes modulates FALDH expression, the effect was tested by treating macrophage cells with high glucose and analyzed the FALDH expression by northern blot. The results showed that there is a significant downregulation of FALDH expression in hyperglycemic condition, suggesting that FALDH might play a key role in protecting cells from the harmful effects of high glucose found in insulin resistance. Since the results showed that hyperglycemia downregulated FALDH expression level, and simultaneously hyperglycemia generated ROS, it was important to determine whether this modulation of FALDH expression is mediated by ROS generation. Previous studies have shown that inhibitors of oxidative stress improve insulin signaling via

suppression of ROS (Jacob et al., 1999, Evans et al., 2002). To confirm that ROS modulates the expression of FALDH in macrophages, cells were co-treated with high glucose and insulin or ascorbic acid, a well known antioxidant, which quenches ROS and protects the cells from the harmful effects of oxidative stress. The results showed that co-treatment of hyperglycemia treated cells with insulin or ascorbic acid reduced ROS production and simultaneously inhibited the downregulation of FALDH expression suggesting that the hyperglycemia mediated FALDH modulation is indeed mediated through ROS. This observation was further confirmed in an experiment where macrophage cells were treated with H_2O_2, a known inducer of oxidative stress and analyzed for FALDH expression. The results showed a significant downregulation of FALDH mRNA expression similar to that observed with high glucose treatment. These data indicate for the first time that hyperglycemia induced oxidative stress mediated through ROS results in downregulation of FALDH expression in macrophages, which can be reversed by treatment with antioxidants or insulin.

The molecular mechanism responsible for decreased FALDH expression in oxidative stress is unknown but could reflect changes in proinflmmatory cytokines associated with insulin resistance. Therefore it might be possible that increased production of inflammatory cytokine like TNFα, MCP-1 and MMP9 could be playing a role. Studies in several diabetic microvascular and macrovascular models demonstrate that hyperglycemia increases the production of various cytokines like TNF-α and chemokines MCP-1 by activation of NFκB pathway in macrophages.

Increased TNF-levels are known to cause insulin resistance through the activation of different serine/threonine kinases, MAPK (JNK, ERK, and p38) as well as IKK (Baynes et al., 1991, Guha et al., 2000, Fujishiro et al., 2003, Nigro et al., 2006). Since hyperglycemia induces TNFα secretion, and increased TNFα levels are known to cause insulin resistance, the role of TNFα in the regulation of FALDH expression in RAW macrophages was studied. RT-PCR analysis showed that FALDH expression is downregulated upon TNFα treatment which indicates that hyperglycemia induced oxidative stress upregualtes TNFα expression which inturn leads to inhibition of FALDH expression.

Next, regulation of FALDH gene was studied in response to hyperglycemia in animal models of diabetes because there have been reports of increased aldehydes and protein adducts accumulation in the aorta of diabetic patients and animals (Thukkani et al., 2003, Oya et al., 1999). Protein adducts and aldehydes are known to play an important role in the development of atherosclerosis and increased accumulation of these products might be due to the downregulation of detoxifying system. Thus the regulation on FALDH gene expression was checked in the aorta of BALBc mice treated with STZ (which specifically destroys pancreatic β cells (Junod et al., 1969, Kunjathoor et al., 1996), and impairs insulin production leading to sever hyperglycemia and oxidative stress). The results show a significant reduction of FALDH mRNA expression in aorta of STZ treated mice as compared to normal mice. In contrast treatment of STZ mice with ascorbic acid in water resulted in

reversal of FALDH expression confirming the role of ROS in regulating FALDH expression. Moreover, the role of ROS in downregulating FADH expression was further confirmed when the result showed a significant reduction in the expression of antioxidant enzyme catalase upon ascorbic acid treatment in the aorta of STZ treated animals. The restoration of FALDH expression in ascorbic acid treated animals might be due to two possible reasons. (1) ascorbic acid might be protecting the β cells from deterioration by STZ, since previous literature has shown that STZ increases oxidative stress in the β cells and causes apoptosis of β cells. A second possible explanation might be that the β cells that are protected from the harmful effects of STZ t are producing insulin and regulating the FALDH expression. To support the second hypothesis, significant improvement in body weight and reduction in the glucose levels were observed in ascorbic acid treated STZ animals as compared to STZ alone treated animals (Data not shown).

Next FALDH expression was studied in the aorta of insulin resistant *db/db* mice which were 12 weeks of age and have very high glucose levels. *db/db* mice, represent a model of monogenic obesity, where the animals are associated with a severe insulin resistant state and high glucose levels (Lee et al., 1996, Chen et al., 1996). The results showed that expression of FALDH mRNA is downregulated in the aorta of *db/db* mice similar to what is been observed in STZ treated BALBc mice. These results indicate that FALDH downregulation observed in aorta of diabetic condition is mediated through hyperglycemia induced ROS and increased

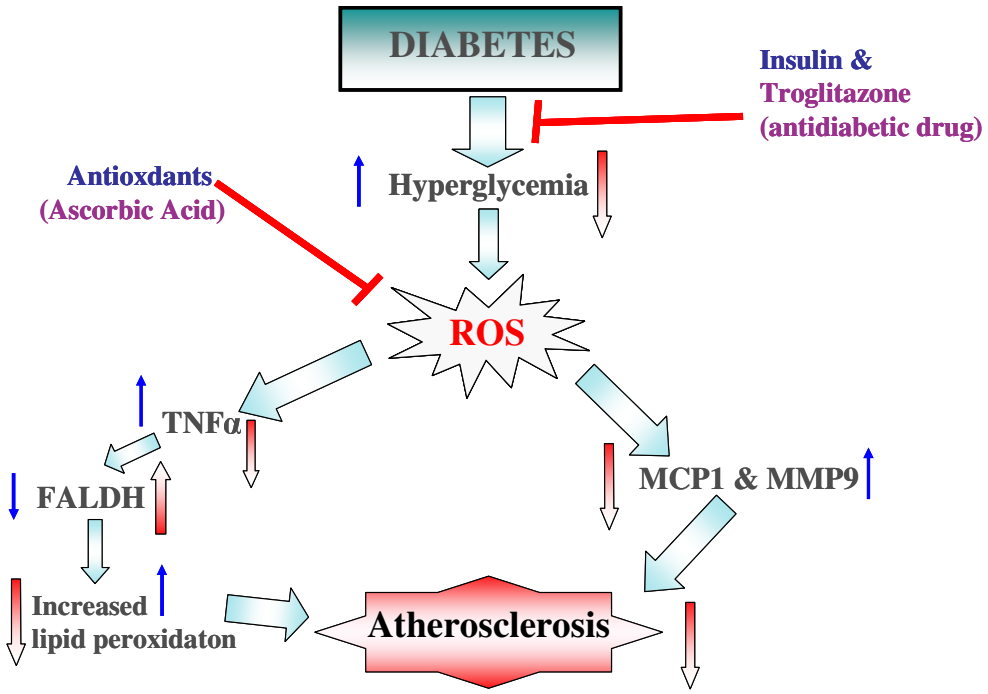
accumulation of aldehydes in the aorta is due to the down regulation of FALDH enzyme function.

In many situations the modulation seen at the transcriptional level is not reflected at translational level, which might be due to some post transcriptional modification. Similar observation has been reported in case of FALDH by Demozay et al., where there was no correlation between the data seen at mRNA level and FALDH enzyme activity in the liver (Demozay et al., 2004). Thus, to confirm whether this is the same in the macrophages, FALDH regulation at the protein level was checked by proteomic analysis. Protein modulation could not be verified by western blot due to non availability of anti-FALDH antibody. FALDH 2D gel analysis is been reported for the first time as no previous literature has been reported as of yet. The data showed that when hyperglycemic condition is treated with insulin, ascorbic acid or troglitazone (antidaibetic drug) there was a reversal of FALDH expression. Troglitazone; an antidiabetic drug was used because it has good insulin sensitization property along with antioxidant properties. The antioxidant property in troglitazone is due to the trolox moiety in its structure. These results show perfect correlation between results obtained at the mRNA level and protein level of FALDH. The data obtained at the protein level confirms that FALDH is downregulated due to hyperglycemia induced oxidative stress and can be reversed by using antioxidants and insulin sensitizing agents.

In conclusion, the results obtained from the *in vitro* and *in vivo* studies suggest that the downregulation of FALDH expression observed at the

transcriptional and the translational level is mediated through hyperglycemia induced oxidative stress (ROS). The suggested mechanism is that, elevated levels of proinflammatory cytokine TNFα induced by oxidative stress resulting in the reduction of FALDH activity **(Fig. 15)**. Since FALDH is an important detoxifying enzyme, its reduced activity might be one of the reasons that result in increased accumulation of aldehydes in the aorta. Thus study of FLADH expression level can be used as a marker to study development of atherosclerosis in diabetic patients. Further studies need to done to evaluate the specific role of FALDH in development of diabetes associated CVD which will enable us to develop novel therapeutic strategies to combat diabetic complications

Figure 15. Propose Mechanism for Downregulation of FALDH in Diabetes.



- Cardiovascular complications characterized by endothelial dysfunction and accelerated atherosclerosis are the leading cause of mortality and morbidity associated with diabetes.

- Diabetes mellitus is characterized with abnormalities like hyperglycemia, dyslipidemia (elevated triglycerides, low high density lipoproteins and increased low density lipoproteins), insulin resistance, obesity and hypertension.

- Hyperglycemia induces a large number of alterations at the cellular level and leads to the generation of highly reactive free radicals as reactive oxygen species (ROS) and reactive nitrogen species (RNS) through various mechanisms which ultimately cause oxidative stress. Increased production of ROS, exceeding the ability of the body to combat it, is considered oxidative stress.

- Overproduction or insufficient removal of these free radicals results in vascular dysfunction, damage to cellular proteins, membrane lipids and nucleic acids.

- Generation of oxidative stress was studied in macrophages, because macrophages are the cells that are involved in the generation of ROS, release inflammatory mediators and uptake oxidized-LDL upon stress, which collectively contribute to atherosclerosis development.

- Incubation of macrophages in high glucose condition increased the generation of ROS. Increased generation of ROS by hyperglycemia was reduced by treating the cells with various antioxidants. Hyperglycemia induced oxidative stress in macrophages mimics an *in vitro* model of diabetes for oxidative stress.

- Insulin regulates the FALDH mRNA expression levels in macrophages similar to what has been reported previously in liver and adipose tissue. This study reports for the first time that FALDH is expressed in macrophages and treatment with insulin increases FALDH expression dose dependently.

- Hyperglycemia resulted in downregulaton of FALDH expression, when macrophages were treated with high glucose condition. Hyperglycemia induced oxidative stress resulted in the induction of proinflammatory cytokine TNFα, which inturn mediated the downregulation of FALDH mRNA expression in macrophages.

- The downregulation of FALDH observed in hyperglycemic condition was reversed when macrophages were co-treated with insulin and antioxidant L-ascorbic acid in hyperglycemic condition. This result confirms that the down regulation of FALDH observed during hyperglycemic condition is mediated through ROS.

- The downregulation of FALDH was observed in the aorta of STZ treated type 1 diabetic and *db/db* type 2 diabetic mice. Treatment of STZ mice with ascorbic acid resulted in reversal of FALDH expression to normal. The role of ROS in regulating the expression of FALDH was confirmed with a decrease in the mRNA expression of antioxidant enzyme catalase in the aorta of STZ + ascorbic acid treated mice.

- The modulation of FALDH in macrophages was checked at the protein level by proteomics. High glucose condition resulted in downregulation of FALDH protein expression in macrophages which was reversed when treated with insulin, antioxidant ascorbic acid and antidiabetic drug troglitazone. Modulation of FALDH at the protein level is been reported for the first time and the result obtained is similar to what is been observed at the mRNA level.

- In summary the results indicate that hyperglycemia induced oxidative stress is up regulating the expression of proinflammatory cytokine TNFα. Increased

induction of cytokine by hyperglycemia might be causing insulin resistance and down regulating the expression of FALDH enzyme. The downregulation of FALDH expression in the aorta of diabetic animals might be responsible for the increased accumulation of aldehydes which are formed as by products of lipid peroxidation.

- The present study is the first systematic investigation that describes the mechanism involved in the downregulation of FALDH in diabetic condition. FALDH being an important detoxifying enzyme and regulated by insulin, it can be used as a marker to study the development of atherosclerosis in diabetic patients.

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