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Pathophysiology of Nonalcoholic Fatty Liver Disease
Endocrinal Profile and Metabolic Pathways

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is now recognized as a common form of chronic liver disease, often associated with the triad of obesity, type 2 diabetes mellitus (T2DM), and dyslipidemia. As the triad essentially characterizes metabolic syndrome, NAFLD may be considered as its hepatic manifestation. Indeed, NAFLD includes spectrum of liver lesions that may represent the hepatic component of metabolic syndrome. Likewise, insulin resistance which constitutes the pathogenic key in metabolic syndrome, also plays a major contributing role in the pathogenesis of NAFLD (1). Although insulin resistance is recognized as one of the major mechanisms involved in disease prevalence and progression, several issues concerned with the pathophysiology of NAFLD remain to be resolved definitively.

Introduction

Hepatic fat accumulation is a well-recognised complication of diabetes mellitus, although associated obesity in T2DM has previously been considered a confounding variable. Recent definition(s) of metabolic syndrome in which impaired fasting glycemia (IFG) and/or impaired glucose tolerance (IGT) are recognized constituents, have brought into sharper focus the causative role of insulin resistance. Several aspects of lipid metabolism including increased transport

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of free fatty acids to liver, enhanced hepatic lipogenesis, and decreased lipid oxidation in the liver, are all related to insulin resistance. Although excessive fat accumulation in the liver is observed in several clinical conditions including prolonged parenteral nutrition, protein malnutrition, jejunoileal bypass and chronic illnesses characterized by impaired nutrition such as ulcerative colitis and chronic pancreatitis, pathophysiology of nonalcoholic fatty liver disease associated with one or more factors including obesity, T2DM, IFG, IGT, and dyslipidemia is distinctly related to metabolic pathways involving insulin resistance.

Prevalence and Causative Mechanisms:

Estimated prevalence of NAFLD in developed countries ranges between 10-24% in *general* population (2). This prevalence rate closely corresponds to the estimated prevalence of 26.7% of metabolic syndrome in US adult population (3). Nevertheless, it is noteworthy that in *obese* populations NAFLD may affect upto 75% of subjects (4). Prevalence data from India have been reviewed (5). The criteria, caveats, and controversies in the diagnosis of metabolic syndrome have been recently reviewed by us (6), along with the methods and measurements of insulin resistance (7). The major focus in the present review would be on the mechanisms underlying fat accumulation in the liver and relating them to the endocrinal profile that affects metabolic pathways. Essentially such mechanisms may include:

i) Excess intake of dietary fat

ii) Excess energy (caloric) intake with low energy expenditure (Life style induced obesity)

iii) Increased delivery of free fatty acids (FFAs) to liver either due to (a) excess lipolysis or (b) inadequate fatty acid oxidation

iv) Increased *de novo* lipogenesis in liver

The most characteristic feature of the pathogenesis of NAFLD, both histologically and metabolically, is the accumulation of triacylglycerol (TAG) in the hepatocyte. The factors (i) – (iv) listed above need to be discussed in contextual perspective.

Excess Intake of Dietary Fat:

Dietary fatty acids may reach liver by either of the two pathways: (i) through the uptake of intestinal chylomicron remnants and / or (ii) through spillover into the plasma FFA pool. Recent increase in the prevalence of obesity, T2DM and dyslipidemia suggest the involvement of metabolic pathways including those resulting directly from insulin resistance and leading to dysregulation of adipose-derived fatty acid flux in the fasting state. In addition, there is strong evidence that in NAFLD patients, insulin suppression of lipolysis in the adipocytes is significantly less than in normal subjects (8). Thus a greater rate of lipolysis contributes higher amounts of FFA to the circulating pool. As the rate of lipolytic activity is higher in the abdominal visceral adipocytes, subjects with visceral (trunkal) adiposity have

higher levels of FFA in portal vein, in addition to daylong elevation of FFA in peripheral plasma. This results in higher amounts of FFA reaching the liver. Using ingenious experimental design, Donnelly *et al.* (2005) (9) have recently quantified the biological sources of hepatic and plasma lipoprotein TAG in subjects with NAFLD who were put on a controlled diet containing 30% of calories from fat. Of the TAG accounted for in liver, nearly 60% of TAG arose from circulating FFA pool; approximately 25% from *de novo* lipogeneis (DNL); and about 15% from the dietary source. In contrast, DNL contributed only about 5% triacylglycerol in a historical control group. *These results suggest that DNL in liver is significantly increased in NAFLD*. Acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) are the major enzymes regulating DNL in liver. Both these enzymes are upregulated by insulin and ghrelin and downregulated by adiponectin.

In spite of some inherent limitations of the study by Donnelly *et al.*, there is a clear message that efforts to treat NAFLD must include strategies aimed at improving insulin sensitivity so as to reduce fatty acid flux, coupled with interventions to modulate hepatic lipogenesis through dietary and pharmacological means. As simple (refined) sugars also stimulate lipogenic pathway, the medical nutrition therapy must include replacement of foods rich in refined carbohydrates with those consisting of complex carbohydrates.

Reduced Fatty Acid Oxidation :

What is the contribution of reduced fatty acid oxidation in the pathogenesis of NAFLD? The rate of hepatic FFA uptake is unregulated and therefore directly proportional to plasma FFA concentration. FFAs taken up by the liver are metabolized by one of two pathways: *oxidation* in the mitochondria to generate ATP or *esterification* to produce triglycerides, which may either be incorporated into VLDL particles, or stored within the hepatocyte. Defects in one or both of these pathways can lead to hepatic steatosis (10,11).

Chronically elevated plasma FFA can lead to insulin resistance in muscle and liver. High FFA concentration may affect several upstream proteins in the insulin signaling pathway, including IRS-1 (Insulin receptor substrate-1) and protein kinase C theta (PKC è) (12). It has been proposed that elevated FFA may activate PKC è, with the resulting decrease in IRS-1 tyrosine phosphorylation, a suppression of PI 3-kinase (phosphatidylinositol 3-OH kinase) activity, reduced GLUT (glucose transporter) translocation, and culminating in a reduction of glucose transport (13). There is evidence to suggest that tyrosine phosphorylation of IRS-1 and IRS-2 (localized in liver) was reduced alongwith blunting of protein kinase-B activation, probably due to the activation of protein kinase C-å, resulting in binding of c-Jun N-terminal kinase (JNK)-1 to IRS-1 and IRS-2 (14).

Additional evidence for the role of intracellular lipid in mediating insulin resistance has been obtained from transgenic mice with muscle-specific and liver-specific overexpression of lipoprotein lipase (LPL). Muscle-LPL-overexpressing mice had a threefold increase in muscle triglyceride content and were insulin resistant due to (i) decrease in insulin-stimulated glucose transport and (ii) a reduced insulin activation of IRS-1 associated PI 3-kinase activity. In contrast, liver-LPL-overexpressing mice had a two-fold increase in liver triglyceride content. These mice were insulin resistant primarily due to the impaired ability of insulin to suppress endogenous glucose production(15). These defects in insulin signaling (and action) were associated with increases in intracellular fat metabolites (i.e. diacylglycerol; fatty acylcoenzyme A). Thus there is a causal relationship between intracellular accumulation of fat metabolites and insulin resistance mediated through changes in insulin signaling pathway.

Hepatic gluconeogenesis and de novo lipogenesis :

Insulin resistance results in hyperinsulinemia which aims at maintaining blood glucose within normal limits as long as possible. The *degree* of insulin resistance *and* the *capacity* of â cells to increase insulin secretion, are the key determinants of glucose homeostasis. When the degree of insulin resistance exceeds the capacity of â cells to maintain compensatory hyperinsulinemia, hyperglycemia begins to manifest. It is in the context of hyperinsulinemia and hyperglycemia that hepatic lipid metabolism needs to be critically reviewed.

In liver, hyperinsulinemia induces the expression of membrane-bound transcription factor, SREBP-1c (sterol regulatory element binding protein-1c) which in turn leads to the transcriptional activation of all *lipogenic* genes in the nucleus (16, 17). Importantly, overexpression of SREBP-1c in transgenic mouse liver results in the development of fatty liver due to enhanced lipogenesis (18). Simultaneously, hyperglycemia activates a second transcription factor, carbohydrate response element binding protein (ChREBP), which transcriptionally activates L-PK (liver type pyruvate kinase) and also all the *lipogenic* genes (19). Activation of L-PK stimulates both glycolysis and lipogenesis. The synergistic activation of SREBP-1c and ChREBP coordinately activates the biochemical pathways for the conversion of excess glucose to fatty acids especially under conditions of energy excess. In the setting of insulin resistance, FFAs entering liver from periphery (as a result of increased lipolysis in the adipose tissue) as well as the FFAs derived from DNL are preferentially esterified to triglycerides in the liver. Hepatic steatosis is characterized by the accumulation of triglycerides in both macro and micro vesicles in more than 5% of hepatocytes, which are predominantly located as perivenular hepatocytes.

Mitochondrial dysfunction :

A subnormal ability of muscle to oxidize fatty acids is an important contributor to

the genesis of insulin resistance. Molecular mechanism(s) underlying FFA induced insulin resistance include : (i) the overexpression of extracellular matrix genes and connective tissue growth factor; and (ii) a decrease in the expression of nuclear encoded mitochondrial genes and of PGC-1á (peroxisome proliferator activated receptor ã – coactivator –1á). As PGC-1á is the transcriptional coactivator that initiates the expression of several genes coding for mitochondrial proteins, a decrease in PGC-1 expression may result in decreased expression of a number of metabolic and nuclear encoded mitochondrial genes involved in electron transport and oxidative phosphorylation (20). Indeed, a decreased expression of nuclear encoded mitochondrial genes, accompanied by a decreased expression of PGC-1á, has been demonstrated in insulin resistant subjects (Fig. 1) (1, 7).

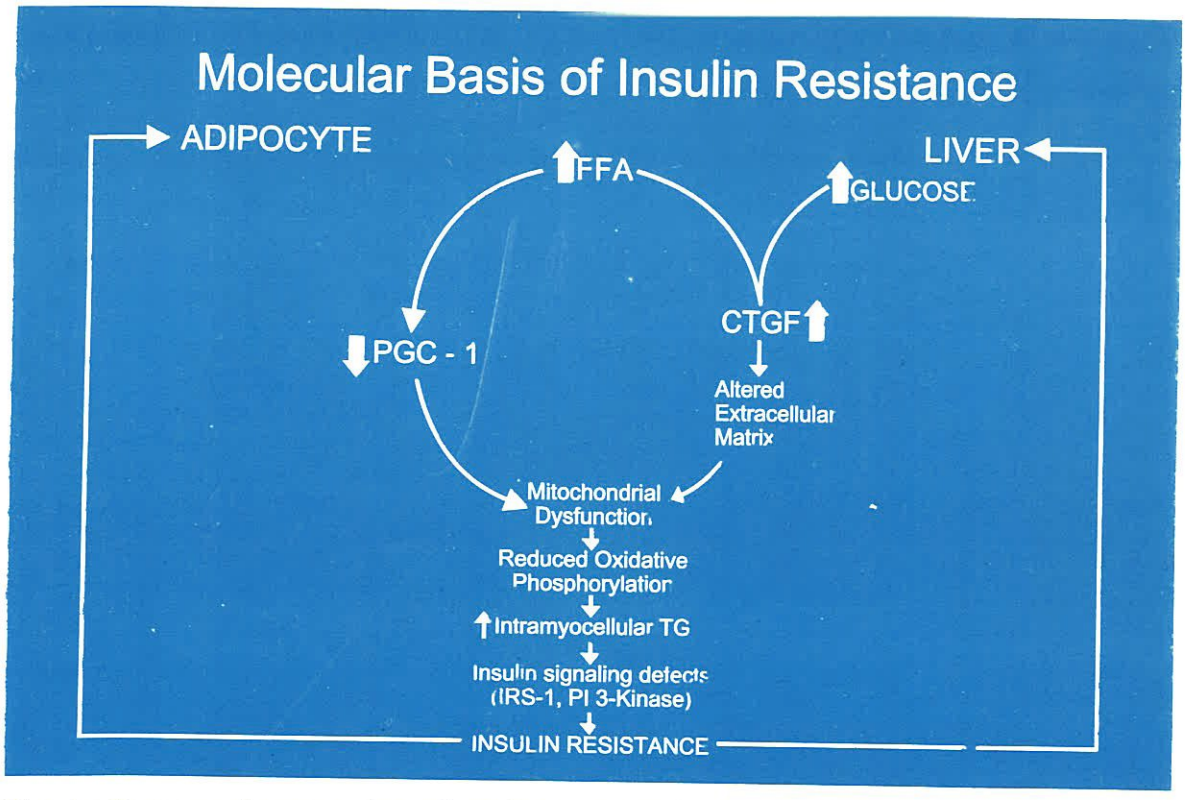

Fig. 1 : Decreased expression of nuclear encoded mitochondrial genes and increased expression of extracellular matrix-related genes may contribute to the molecular basis of insulin resistance (for explanation, see text). From Bajaj and Bajaj (1, 7) with permission of Editor and Publisher

PGC - 1α : peroxisome proliferator activated receptor γ - coactivator - 1α; CTGF : connective tissue growth factor; FFA : free fatty acids; IRS-1 (insulin receptor substrate 1); PI 3-Kinase (phosphatidylinositol 3-OH kinase)

Notwithstanding the effect on nuclear encoded mitochondrial genes, a marked over expression of matrix-related genes in response to elevated FFA characterizes inflammatory response leading to extracellular matrix remodeling and fibrosis. Thus chronic elevation of FFA may result in inflammation-associated extracellular matrix changes in the skeletal muscle. Such fibrotic inflammatory responses are mediated by the connective tissue growth factor (CTGF), also termed CCN2, a 38KDa protein belonging to the CNN family (21). There is evidence that CTGF mediates fibrotic changes at multiple sites i.e. atheromatous plaques; mesangium in the glomerulus; myocardium following ischemic injury and activated hepatic stellate cells(22) (Fig.2) (1, 7) . Of major interest is the observation that liver biopsis from nondiabetics and T2DM patients with non-alcoholic steatohepatitis (NASH) showed enhanced expression of CTGF that correlated with the degree of fibrosis (22). As the CTGF expression is increased in the liver from Zucker obese rats in association with lipid abnormalities and fatty liver in this animal model of insulin resistance, it

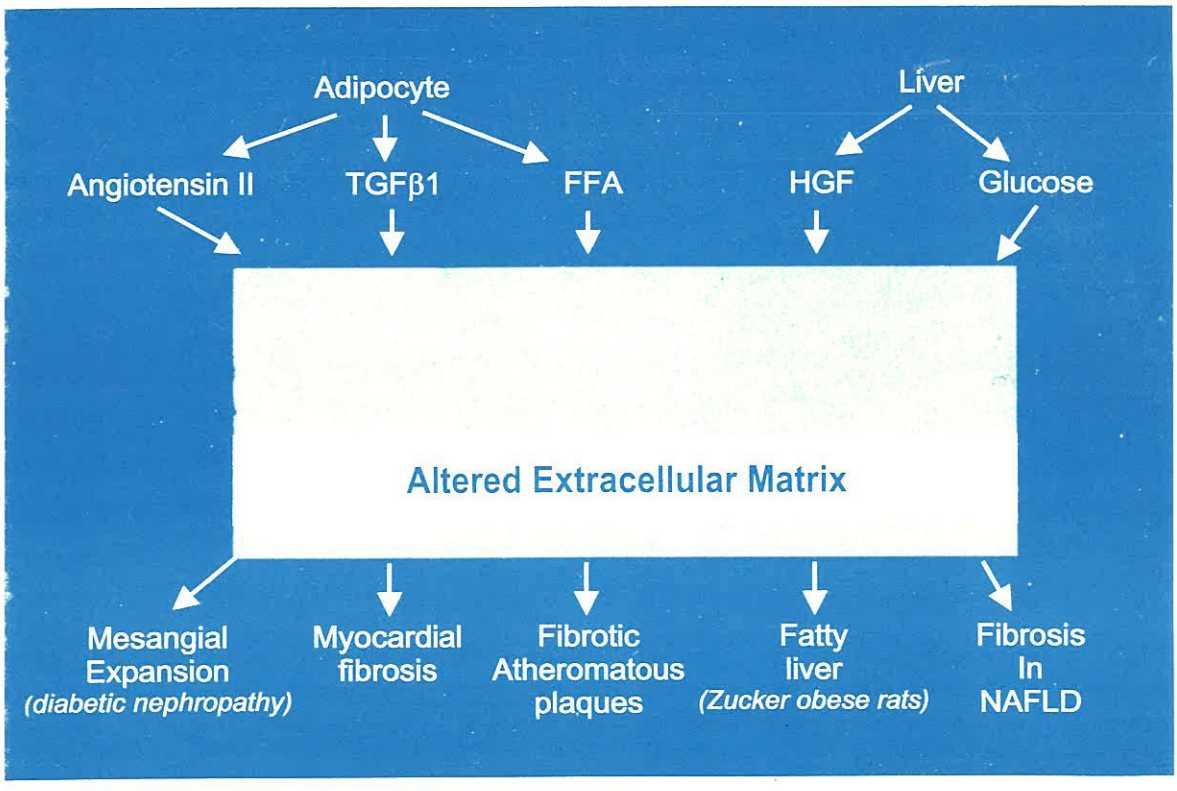

Fig. 2 : Enhanced expression of connective tissue growth factor and its possible consequences. From Bajaj and Bajaj (1, 7) with permission of Editor and Publisher.

may be a rational molecular target for future drug development. Indeed, an angiotensin II type 1 receptor antagonist, olmesartan medoxomil, has been shown to improve experimental liver fibrosis by suppression of proliferation and collagen synthesis in hepatic stellate cells(23).

Adipocyte Endocrinology, Cytokines, Inflammation, and Insulin Resistance:

A major advance in adipocyte biology in recent years is the elucidation of molecular pathways that link inflammation and insulin resistance. The concept of fat as a site of production of cytokines, initiated by the early work on proinflammatory cytokine TNF-á, has now been extended to include leptin, resistin, IL-6, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, retinol binding protein-4, serum amyloid A (SAA) and others. Essentially the triad of resistin, TNF-á and IL-6 constitute the proinflammatory cytokines that primarily induce insulin resistance. They also, alongwith other proinflmmatory cytokines, seem to be involved in the induction of subacute inflammatory state associated with obesity. They play a significant role in the recruitment of macrophages to adipose tissue. Furthermore, they activate intracellular pathways that facilitate the development of insulin resistance and T2DM. Obesity and high fat diet are associated with higher levels of TNF-á, which activates Inhibitory êB-Kinase-â (IKKâ). This in turn degrades IKB and allows activation and nuclear migration of nuclear factor (NF)-êB, leading to upregulation of IL-6 (24). TNF-á also activates JNK-1 (25). The whole inflammatory pathway, IKKâ / NF-êB and JNK-1, is thus activated in the adipocyte (26). In contrast, adiponectin expression correlates inversely with serum TNF-á levels (27) : adiponectin is the *only* adipokine which enhances insulin sensitivity and is antiinflammatory and anti-atherogenic (*vide infra*).

It is easy to extrapolate the role of inflammatory pathways in the adipocyte, as detailed above, to the hepatocyte. Most significantly, the same pathways, as activated in the adipocyte, are also activated in the hepatocyte with increasing steatosis. Obesity induced JNK activation promotes the phosphorylation of IRS-1 at *serine* sites that negatively regulate normal signaling through the insulin receptor-IRS-1 axis where phosphorylation at tyrosine sites is facilitated by tyrosine kinase. Finally, systemic markers of oxidative stress as a result of increasing adiposity, bring in the role of reactive oxygen species (ROS) in the development of obesity-induced insulin resistance (28, 29). ROS initiate lipid peroxidation, resulting in the formation of long half-life aldehyde by-products including 4-hydroxy-2 nonenal (HNE) and malondialdehyde (MDA). These compounds, in addition to propagating

oxidative damage, induce cellular alterations including microvesicular steatosis, glutathione depletion, and enhanced TNF-á production; the latter aggravates inflammatory activity, cellular necrosis, and fibrogenesis through enhanced production of TGF-â$_1$.

NAFLD is often associated with abdominal obesity and as outlined earlier, may be a constituent of metabolic syndrome. The pathological spectrum of NAFLD ranges from steatosis to steatohepatitis, progressive fibrosis and finally cirrhosis. Inflammation plays a key role in the progressive changes resulting from accumulation of TAG in the hepatocyte. *They contribute to insulin resistance at the hepatocyte.* Indeed, hepatocyte lipid accumulation as a result of various pathways (illustrated in the first part of this paper), induces a subacute inflammatory response in the liver, similar to the one in the adipose tissue. In addition, proinflammtory cytokines in the portal circulation as a result of excess production in increased abdominal fat, may contribute to, or potentiate, hepatic inflammation. As a result, NF-êB is activated in the hepatocyte and cytokines such as IL-6, TNF-á and IL-1B are excessively produced in the liver, leading to activation of Kupffer cells, the hepatic macrophages. It may be emphasized that while the number of Kupffer cells does not increase with steatosis, yet their state of activation is enhanced.

Molecular Biology of Adiponectin :

Adiponectin is a novel adipose-specific 247-aminoacid protein, with high structural homology to TNF á (30). While pharmacological doses of recombinant resistin hyperactivate gluconeogenesis through decreased hepatic insulin sensitivity, adiponectin inhibits gluconeogenesis by increasing insulin sensitivity. As mentioned earlier, adiponectin is considered as an antidiabetogenic and anti-atherogenic adipokine. Plasma levels of adiponectin are reduced in obese rodents (31) and humans and also in subjects with T2DM(32). It has been suggested that adiponectin might function as an adipostat in regulating energy balance and that its deficiency might contribute to the development of obesity and T2 DM.

Adiponectin and hepatic gluconeogenesis : Intraperitoneal administration of full-length recombinant adiponectin has been shown to produce a decrease in blood glucose, indicating liver as a likely target of adiponectin, a fact later confirmed by *in vitro* studies on isolated hepatocytes (33). Using hyperinsulinemic-euglycaemic clamps it was shown that adiponectin lowers hepatic glucose production without affecting peripheral glucose uptake. The molecular basis of hepatic effects of adiponectin is through the reduced hepatic expression of glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase

(PEPCK), the gluconeogenic enzymes regulating endogenous glucose production and hepatic glucose output. Thus resistin and adiponectin affect the hepatic expression of key gluconeogenic enzymes in opposite direction : resistin enhances the expression of G6Pase and PEPCK, while adiponectin reduces their expression in the liver.

Adiponectin, hepatic fat, and insulin resistance : Increased hepatic fat content is an important determinant of hepatic insulin resistance in type 2 diabetic patients (34). As fatty liver is common in such patients, it has been suggested that fatty liver results from accelerated fatty acid mobilization from expanded visceral fat stores and their deposition in the liver, as well as decreased hepatic fatty acid oxidation thereby causing an increase in the hepatic fat content. Thiazolidinediones have been shown to reduce hepatic fat content and improve hepatic insulin sensitivity in patients with T2DM (34). The thiazolidinediones initiate their action by binding PPARã, primarily located on adipocytes (35), and thereby increasing plasma adiponectin levels. Indirect evidence suggests that adiponectin might mediate some of the insulin-sensitizing effects of PPARã agonists.

The first clinical study aimed at investigating the effect of long term (14 weeks) administration of 45 mg. pioglitazone daily in subjects with T2DM resulted in a three-fold increase in plasma adiponectin which correlated inversely with endogenous (hepatic) glucose production. There was also a significant inverse correlation of plasma adiponectin with hepatic fat content. Higher the plasma adiponectin levels, lower the hepatic fat content. Thus the increase in plasma adiponectin following pioglitazone therapy is strongly associated with a decrease in hepatic fat content and enhanced hepatic and peripheral insulin sensitivity (36).

Put together with a study on resistin undertaken by the same investigators, wherein a significant decrease in resistin levels was shown following pioglitazone administration, there is unequivocal evidence that pioglitazone (thiozolidinedione) treatment of subjects with T2DM increases plasma adiponectin and decreases plasma resistin levels, resulting in a decrease in hepatic fat content and a reduction in hepatic glucose production. Indicators of net therapeutic benefit included a decrease in fasting plasma glucose as well as a lowering of the HbA1c and serum triglyceride levels (36).

Therapeutic Implications and Applications :

In addition to the effects on metabolic pathways, thiozolidinediones class of PPAR-ã agonists exhibit antiinflammatory properties, which are also shared by statins that inhibit HMG CoA reductase (37). Both class of drugs therefore provide potential

benefits beyond their primary action on glucose homeostasis and lipid metabolism, respectively.

The primary mode of action of TZDs is through binding and activation of PPAR-ã at the adipocyte. But in addition to adipocyte, PPAR-ã is also expressed in macrophages and other immune cells, hepatocytes, endothelial cells and vascular smooth muscle cells (VSMCs). The antiinflammatory action of the TZDs may be mediated through the transrepression of NF-êB and consequent decreases in the expression of target genes for cytokines and growth factors, cell proliferation, and migration (38). Another possibility, albeit remote, is that TZD may exert antiinflammatory effects through PPAR-ã-independent activation of glucocorticoid receptor (39).

As in the case with TZD, several of the statins in clinical use have been shown to downregulate transcriptional activities of NF-êB with concomitant reduction in the expression of inflammatory cytokines. Clinical trials evaluating statins have shown reduction in circulating levels of CRP, multiple cytokines, and inflammatory markers (40).

As pathogenesis of NAFLD involves complex interplay of insulin resistance and inflammation, attempts have been made to enhance insulin sensitivity through both non-pharmacological and pharmacological interventions. The former include reduction of body weight, both by reducing energy intake (quantitative) as well as by reducing the contribution of energy-dense saturated fats to total energy intake (qualitative). In addition, as mentioned earlier, refined sugar intake must be curtailed as a part of medical nutrition therapy which should include high fiber diet. Added to this, lifestyle interventions including enhanced day-to-day physical activity, a regular exercise regimen, no smoking, and complete abstinence from alcohol, have shown beneficial effects. Physical exercise, even moderate in intensity, if undertaken regularly, increases the oxidative capacity of myocytes and enhances oxidation of fatty acids (41). A decrease in myocellular lipid enhances insulin sensitivity (42). It is well demonstrated that intensity of aerobic physical exercise is an important determinant of insulin sensitivity in the muscle. Nevertheless, in a well designed study, even moderate intensity aerobic exercise performed by previously sedentary men and women resulted in a significant improvement in serum aminotransferases in non-alcoholic steatohepatitis (43).

Pharmacological interventions by the use of insulin sensitizers such as TZD have already been mentioned. Metformin is safe insulin sensitizer. It activates AMP kinase which results in reduction of hepatic glucose production, decreased lipid synthesis, *as well as* increased fat oxidation (44). Initial clinical trials have provided

equivocal results. There is a need for randomized, placebo-controlled trials before metformin is recommended as a part of therapeutic armamentarium in NAFLD. Likewise, the role of antioxidants and other hepatoprotective agents remains to be established though rigorously controlled clinical trials.

Epilogue :

Future research is likely to provide additional information on the role of genetic and environmental factors, especially the *in-utero* protein undernutrition and metabolic environment which may determine the development of insulin resistance, metabolic syndrome, type 2 diabetes mellitus (45), and NAFLD in adult life. Indeed, if such a mechanism is confirmed, then preventive measures must start with emphasis on maternal nutrition during pregnancy. A continuum of preventive strategies must include lifestyle changes with emphasis on balanced nutritional and physical exercise which should be a part of school health programmes, and subsequently continued through adult life.

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