

## INSULIN RESISTANCE IN NASH

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### 1. ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease observed in the clinical practice of hepatology. The coexistence of metabolic syndrome in this cohort of patients has made insulin resistance central to the pathogenesis of these disorders. The metabolic consequence of insulin resistance is impaired hepatic glucose output and abnormal lipid handling. In the face of continued metabolic insults the normal hepatic regulatory mechanism gets overwhelmed and fat accumulates in the hepatocytes. The subsequent fate of steatotic hepatocytes depends on the capacity of additional factors such as adipocytokines and toxicity induced by the free fatty acids themselves to induce inflammatory response. This latter process is responsible for the producing the phenotype of non-alcoholic steatohepatitis (NASH). Irrespective of the process by which these phenotypic response occurs, it is now universally accepted that in the absence of insulin resistance the spectrum of changes one associates with NAFLD does not develop. In this review we will discuss the various processes that are involved in the pathogenesis of NAFLD.

### 2. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of liver disorders characterized by macrovesicular steatosis that occurs without alcohol as an inciting cause. The histology of NAFLD extends from a fatty liver (NAFL) to steatohepatitis (NASH). NASH can progress to cirrhosis. The principal risk factors for NAFLD are obesity and diabetes. Data obtained from the NHANES survey suggest that nearly 30% of the United States population are obese (BMI $\geq$ 30) and suffer from one or more complications of obesity (1, 2). It is also well recognized

that obesity is associated with hypertension, coronary artery disease, dyslipidemia, type II diabetes mellitus, increased risk of certain cancers and NAFLD (3-6). The common denominator that is associated with all these obesity-related complications is insulin resistance (IR), and most available evidence points toward its primary role in the pathogenesis of these disorders (Figure 1). It is now well accepted that IR and subsequent hyperinsulinemia may not only be the primary triggers for development of hepatic steatosis but may also be responsible for the progression of fatty liver to steatohepatitis and subsequent fibrosis.

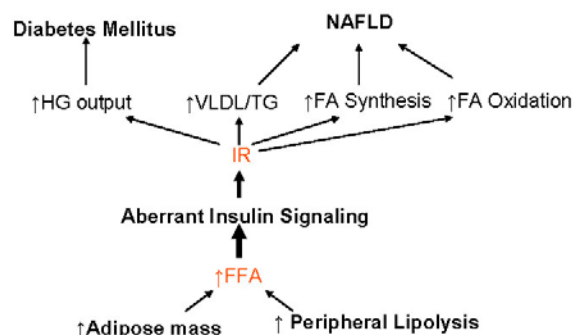
### 3. LINK BETWEEN INSULIN RESISTANCE AND FATTY LIVER DISEASE

Several lines of evidence including clinical observations, epidemiological associations and experimental data indicate that IR plays a central role in the pathogenesis of NAFLD. Patients with NAFLD are commonly obese (BMI $\geq$ 30) and have associated hypertension, dyslipidemia and diabetes, the manifestations of metabolic syndrome (7-9). In addition, fatty liver disease is commonly seen in patients with lipodystrophic disorders that are known to have severe insulin resistance (10). These associations tend to suggest that in the IR state, the liver is one of the target organs that is affected, and that NAFLD may be described as the hepatic manifestation of the metabolic syndrome.

Epidemiologic data obtained from autopsy studies have reported the incidence of fatty liver in 18.5% of obese individuals as well as 2.7% of lean subjects (11). Similar data have been reported in studies conducted on victims of air crashes (12). In addition, the prevalence of an



**Figure 1.** Interplay of Obesity with Metabolic Syndrome in the genesis of NAFLD.



**Figure 2.** Abnormalities in lipid and carbohydrate metabolism in Obesity.

echogenic liver, a finding that is suggestive of NAFLD, has been detected in a high proportion of obese patients (13, 14). It is currently believed that obesity may be associated with 70-80% risk of NAFL and 15-20% risk of NASH (15). These findings indicate that obesity is a strong and independent risk factor for the development of NAFLD.

Several studies have looked at biochemical evidence of IR in patients with NAFLD. These include studies that have demonstrated elevated fasting insulin and C-peptide levels in patients with NAFLD in comparison to age and gender matched controls with other chronic liver diseases (16-18). In addition, non-diabetic precirrhotic individuals with NAFL as well as NASH have been shown to have IR. While these data show a strong association between NAFL and IR, the role of IR in the progression to NASH has remained controversial. Recent data has shown that diabetes is an independent risk factor in the development of fibrosis, and the patients who had more advanced fibrosis had significantly worse glucose tolerance (19). Although more work needs to be done to clarify the various aspects of pathogenesis of NAFLD, it is clear that in the absence of IR, NAFLD rarely develops.

## 4. DEFINING AND MEASURING INSULIN RESISTANCE

Insulin resistance and insulin sensitivity are both measured by the ability of insulin to clear a given amount of glucose from the circulation. IR is measured along a continuous sliding scale for a given individual and varies inversely with insulin sensitivity. As both insulin resistance and sensitivity represents the metabolic ability to clear

glucose from the circulation, there is no threshold value at which IR develops in a given individual and at any point in time, every one is somewhat sensitive and somewhat resistant to insulin.

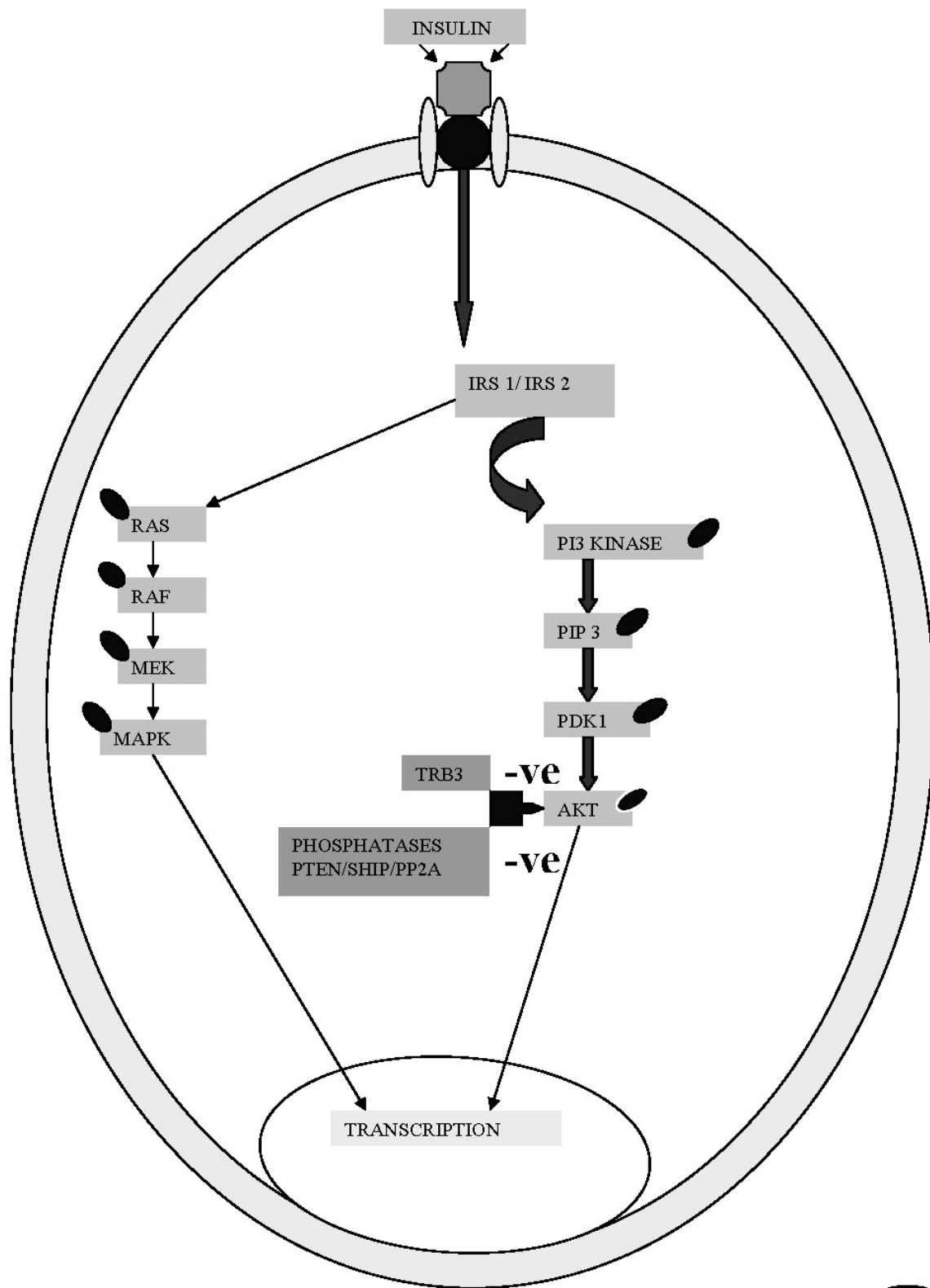
The gold standard for measurement of IR is the euglycemic hyperinsulinemic clamp where insulin is infused at a constant rate while glucose is titrated to maintain euglycemia (20). Thus when there is an increase in insulin sensitivity, more glucose needs to be infused to maintain euglycemia. While this method is very accurate and reflects the rate at which glucose is being transferred from the extracellular space into the cell, it is cumbersome to perform and is not practical for routine clinical applications. Clinically IR is measured most commonly by the HOMA-IR method which calculates the value based on fasting insulin and glucose values (21-24). This method for measurement of IR correlates well with clamp data and is considerably simpler and more convenient to perform. Alternate methods for measuring IR include oral and intravenous glucose tolerance tests along with insulin measurements, all of which are comparable to the clamp method (25, 26).

### 4.1. Physiological and biochemical actions of insulin

The metabolic and physiologic actions of insulin are initiated by the binding of insulin to its receptor. This leads to a complex series of biochemical and molecular events that eventually influences glucose and lipid homeostasis. To understand IR and its role in the development of NAFLD, it is important to clarify the normal physiological actions of insulin.

### 4.2. Insulin signal transduction and related molecular events

The insulin receptor is a hetero-tetramer with two extracellular domains and two membrane spanning domains (27). It belongs to the tyrosine kinase family of receptors that include insulin like growth factor (IGF) receptors, extracellular growth factor (EGF) receptors and insulin receptor related (IRR) receptors (28). Following binding of insulin to the extracellular subunit, there is autophosphorylation of the subunit. Subsequently, there is recruitment and phosphorylation of insulin receptor substrate (IRS) proteins and initiation of a downstream signaling cascade (Figure 3). Additionally, there is also phosphorylation and recruitment of substrates such as Gab-2-associated binder 1 (Gab-1) and Src homology-2 (SH2) proteins containing SH2 domain to the IRS. These proteins have the dual role of both amplifying as well as lending specificity to the insulin induced signal propagation. Downstream activation of specific signal transduction molecules including phosphatidylinositol-3-kinase (PI-3K) and mitogen activated protein kinase (MAPK) involves the mediation of two major adapter proteins (29-31). These include the p85 subunit of PI-3K and growth factor receptor binding protein 2 (Grb-2), both of which are involved in the regulation of PI3-K and MAPK pathways. The IRS/PI3K pathway is predominantly involved in the process of glycogen synthesis and translocation of glucose transporter GLUT-4 to the cell surface to facilitate the entry of glucose into the cell. An activated PI3-K transduces signal



**Figure 3.** IRS – insulin receptor substrate; RAF- mitogen activated protein (MAP) kinase kinase kinase; MEK- MAP kinase kinase; MAPK- MAP kinase; PIP 3- phosphatidyl inositol 1,3 triphosphate; PDK1- phosphatidylinositol dependant kinase 1; TRB3-tribbles homologue. Phosphate group-.

into either protein kinase B (also known as AKT) or 8 and isoforms of protein kinase C (PKC) (32, 33). Insulin induced signal transduction through PI3K/AKT is modulated by tribble-related Drosophila homologue (TRB3) (34). TRB3 is responsible for inhibiting AKT by directly blocking its phosphorylation and thereby preventing signaling downstream of PI3K. This observation is supported by detection of elevated levels of TRB3 in fasting state, allowing for increased glucose output and inhibition of insulin action to prevent hypoglycemia. In the IR state, it has been proposed that TRB3 induced inhibition of AKT allows for increased hepatic glucose output despite the presence of hyperinsulinemia (34). The MAPK pathway is responsible for the modulation of insulin effects on growth and proliferation and acts via intermediaries such as MAPK-kinase (MAPKK) (35).

The G protein related signal transduction pathway is also utilized by insulin. In insulin sensitive tissues Cbl, the product of cbl proto-oncogene and another substrate in the presence of a specific adaptor protein known as cbl-associated protein (CAP) is tyrosine phosphorylated by insulin. This cbl-associated protein is transcriptionally regulated by peroxisome proliferation activator receptor (PPAR-) agonists. The complex of Cbl-CAP translocates to a lipid raft in the plasma membrane and interacts with flotillin and another adaptor protein CrkII (36). CrkII recruits a guanyl nucleotide-exchange protein, C3G which in turn activates the G-protein TC10 (37). This activated G-protein receptor is responsible for modulating the activity of proteins such as syntaxin and other related proteins that facilitate interaction of vesicles with the plasma membrane. The net result of these interactions is exocytosis as well as translocation of GLUT-4 to the cell surface and enhancement of the ability of cells to take up glucose (38, 39).

The effect of insulin on carbohydrate and lipid metabolism is mediated by its action on nuclear transcription factors sterol regulatory binding protein 1c (SREBP-1) and the forkhead family of transcription factors (40, 41). Insulin increases the expression of SREBP 1c and consequently enzymes responsible for fatty acid synthesis including the key enzyme acetyl CoA carboxylase. The increase in SREBP 1c expression by insulin is also responsible for the uptake and the metabolism of glucose by virtue of its effect on both glucokinase and phosphoenol pyruvate carboxykinase (PEPCK) (42, 43).

The termination of insulin action is brought on by regulatory elements such as phosphotyrosine phosphatase (PTP) as well as end product feedback (31, 44). PTP is responsible for dephosphorylation of phosphorylated tyrosine residues, thus serving to terminate insulin action. In addition the number of substrates that are recruited by insulin serve as both kinases and phosphatases, thus acting as molecular switches for regulation of insulin action.

### 4.3. Physiological actions of insulin on intermediary and fat metabolism

Hyperinsulinemia and IR are responsible for the core metabolic abnormalities that are observed in NAFLD.

To better understand the pathogenesis of insulin induced hepatic damage, it is important to review the normal biochemical actions of insulin on carbohydrate and fat metabolism.

### 4.4. Carbohydrate metabolism

The effect of insulin on carbohydrate metabolism is at multiple levels including uptake, intracellular processing and ultimate disposal of glucose. Insulin increases cellular uptake of glucose by increasing the expression of glucose transporter (GLUT) to the cell surface. This is achieved by both increased translocation of GLUT containing vesicles to the cell surface and to a lesser extent by decreased rate of internalization of the receptor (45). Following cellular uptake, the metabolic fate of glucose depends on the nutritional status of the individual. In the fed state, glucose is converted to glycogen in the presence of the enzyme glycogen synthetase (GS). GS forms a macro-molecular complex with glycogen phosphorylase and protein phosphatase-1 (PP-1) (46). These two enzymes are closely associated with GS and act as molecular triggers keeping GS in an inactivated state (47). Insulin controls this process by promoting glycogen synthesis and inhibiting glycogenolysis by its action on protein kinase C via the IRS/PI3k pathway (48). In addition, insulin also increases intracellular pools of PP-1 that serve as scaffolding for formation and elongation of glycogen molecules.

In a state of negative carbohydrate balance, the metabolic state tilts towards glycolysis and glycogenolysis. Substrate availability is also increased by mobilization of glucose from the extracellular space and increased gluconeogenesis. In the glycolytic pathway following cellular uptake, glucose is phosphorylated to glucose-6-phosphate in the presence of the enzyme hexokinase. Subsequently glucose-6-phosphate is converted to fructose-6-phosphate which is then quickly phosphorylated to fructose-1,6-bisphosphate (F-1,6-P). This is the rate limiting step of glycolysis and is catalyzed by the enzyme phosphofructokinase (PFK-1). PFK-1 is allosterically activated by cyclic adenosine monophosphate (cAMP) and inhibited by adenosine triphosphate (ATP). It is also subject to inhibition by citrate and fructose-2,6-bisphosphate, a product of the reaction phosphofructokinase-2/fructose 2,6-bisphosphatase. The subsequent metabolic fate of glucose depends on the availability of oxygen. Under aerobic conditions glucose eventually enters the Krebs' cycle and energy is generated via the mitochondrial electron chain by phosphorylation of adenosine diphosphate to ATP. When there is a low supply of oxygen, pyruvate is converted to lactate with resultant decreased formation of ATP. Insulin acts on the rate limiting enzymes in the process of glycolysis by its action on protein kinase A via IRS/PI3K pathway (48). Glucagon and insulin have opposing effects on PKA and the insulin/glucagon ratio determines whether carbohydrates are stored or metabolized. When this ratio is high the reaction favors dephosphorylation and activation of PFK with increased production of F-2,6-P. This is reversed when the ratio is low and PKA is activated.

Glucose can also be produced during the

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interdigestive period from amino acids such as alanine. Alanine is converted to oxaloacetate which is then converted to phosphoenol pyruvate. This step is catalyzed by the enzyme phosphoenol pyruvate carboxykinase (PEPCK), the genetic transcription of which is directly inhibited by insulin (49). Thus under conditions of carbohydrate excess and consequent high insulin/glucagon ratio, gluconeogenesis is inhibited.

## 5. FAT METABOLISM

The metabolism of fat in the body is dependent on the state of energy balance. In conditions of energy excess, the cellular machinery is directed towards both *de novo* lipogenesis as well as increased conversion of available FFA into triglycerides for storage. FFA absorbed from the diet as well as mobilized from visceral adipocyte storage sites are carried predominantly by the portal vein into the liver. Hence the level of FFA in the portal circulation reflects the extent of lipolytic activity of the visceral adipocytes as well as the extent of dietary absorption. In the liver parenchymal cells, the subsequent fate of FFA is dependent on the net energy balance of the cell. In the fed state with an adequate supply of substrate, the FFA is taken up by the mitochondria, the cytochrome P-450 system or the peroxisomes. In the mitochondria and the peroxisomes, the FFA undergo oxidation while the microsomal system is responsible for  $\beta$  oxidation. While the microsomal system for FFA metabolism is of less importance in healthy individuals, it assumes increased role in presence of excess FFA availability. This diversion of FFA metabolism to microsomes under conditions of substrate excess leads to generation of reactive oxygen species (ROS) and the development of oxidative stress that is observed in patients with NAFLD. Insulin is involved both in the process of lipogenesis as well as in lipid storage. The insulin/glucagon ratio in a given individual determines the extent to which the cellular machinery gets directed towards lipogenesis versus lipid degradation. These metabolic actions of insulin are mediated via regulation of key transcriptional factors SREBP and peroxisome proliferator activated receptor (PPAR) system. The regulation of SREBP is closely linked with the regulation of several key enzymes involved in the process of lipid metabolism including fatty acid synthetase and acyl CoA carboxylase. Hence insulin induced increase in SREBP expression results in increased expression of lipogenic genes and consequent increase in the *de novo* lipogenesis. The reverse effect is observed when there is reversal of the insulin/glucagon ratio.

The PPAR system is also directly involved in the process of regulation of lipogenesis and lipid oxidation. The PPAR system consists of 3 subtypes, PPAR $\alpha$ , PPAR $\beta$ / and PPAR $\gamma$  with distinct tissue distribution and biological actions. In the liver PPAR $\alpha$  predominates and is involved in the regulation of fatty acid catabolism while PPAR $\gamma$  is highly expressed in adipose tissues. Studies have demonstrated that PPAR $\alpha$  directly regulates genes involved in fatty acid uptake [fatty acid binding protein (FABP)], oxidation (acyl-CoA oxidase) and  $\beta$  oxidation (cytochrome P450) (50, 51). The activation of these genes by the PPAR

system leads to a net increase in the disposal of FFA. In the mitochondria, this effect of the PPAR system on the lipid oxidation genes causes activation of carnitine palmitoyltransferase (CPT) and fatty acid transport proteins leading to increased mitochondrial FFA uptake and oxidation. In addition, PPAR system is also responsible for peroxisomal uptake and oxidation of long chain fatty acids ( $C > 20$ ) (52). Hence in summary, the PPAR system senses the circulating fatty acid levels and in times of limited substrate availability reduces hepatic triglyceride accumulation and drives the cellular metabolic machinery towards fat oxidation. Thus it serves as an adaptive response to caloric depletion that is observed during starvation or fasting, generating ketone bodies for energy requirements of the peripheral tissues.

Mitochondrial handling of FFA is dependent on the caloric state of the cell and the insulin/glucagon ratio. In a state of excess availability of glucose and a high insulin/glucagon ratio, there is increased FFA synthesis and accumulation of malonyl CoA in the cytosol. Elevated levels of malonyl CoA inhibit CPT that results in decreased ingress of FFA into the mitochondria. This leads to cytosolic accumulation of FFA with increased formation of VLDL and TG. In calorie starved states with low insulin/glucagon ratio, the situation is reversed. Malonyl CoA levels are reduced leading to loss of CPT inhibition and increased entry of FFA into the mitochondrial  $\beta$  oxidation pathway. The net consequence of this FFA catabolism is the generation of ketone bodies that serve as a source of energy for the peripheral tissues. During the process of  $\beta$  oxidation of FFA in the mitochondria, oxidised co-factors NAD $^{+}$  and FAD $^{+}$  are reduced to NADH and FADH $_2$ . The process of re-oxidation of these cofactors generates electrons that are transported down the electron transport chain. During the process of electron transfer, protons are transported into the mitochondrial intermembrane space creating an electrochemical gradient that is responsible for the generation of energy. Most of the energy that is generated during the process of electron transport is trapped in the phosphate bond of ATP. However, some of the energy is lost during this process as a result of formation of free radicals that are responsible for causing intracellular oxidative stress (53).

Further modulation of fatty acid metabolism is achieved through long chain acyl CoA (LC-CoA). LC-CoA is the first product of fatty acid oxidation and gets metabolized to ceramide, phosphatidic acid diacylglycerol. These by-products are responsible for the inhibition of pyruvate kinase as well as the modulation of insulin activity and metabolic actions (54). In addition, LC-CoA directly increases PPAR activity and contributes to the positive feedback loop that is responsible for fatty acid oxidation in times of caloric deprivation (55).

### 5.1. Mechanisms of insulin resistance

Insulin resistance is a dynamic process that is closely linked with glucose homeostasis. It involves a complex interplay between the pancreatic insulin secretion and the response of target organs to increasing levels of insulin. The development of IR is multifactorial and involves

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**Table 1.** Sites of insulin resistance in animal models

<b>A. Proximal signal effector</b>	
1. Insulin receptor (IR)	
IR -/-	Normal growth in utero, but die from ketosis within 1 week (not seen in human form of hyperinsulinemia-leprechaunism)
IR +/-	Moderate hyperinsulinemia (10% develop diabetes)
2. Insulin receptor substrate (IRS)	
IRS-1 -/-	Mild IR/ impaired glucose tolerance/delayed growth/ diabetes not seen for multifold insulin rise from $\beta$ cell $\beta$ hyperplasia.
IRS-1 +/-	Lack the changes like IRS -/-
IRS-2 -/-	Develop diabetes by 10 weeks of age/reduced $\beta$ cell mass/ resemble human type 2 diabetes
IRS-3 -/- /IRS-3 +/-	Normal phenotype
IRS-4 -/- /IRS-4 +/-	Normal phenotype
<b>B. Downstream signal transducers</b>	
1. Akt2	Insulin resistance in liver/muscle
2. PTP 1B	↑ Insulin sensitivity
3. P85 $\alpha$ (hetero)	↑ Insulin sensitivity
4. SHP2	↑ Insulin sensitivity
5. Glucose transporter (GLUT-4)	↑ Insulin levels/nor glucose

IRS: Insulin receptor substrate, Akt: protein kinase B, PTP: protein tyrosine phosphate, SHP: SH2 containing tyrosine phosphate. Adapted with permission from Sanyal *et al* Clinical Gastroenterology 2002 (16) 5 Page 723

**Table 2.** Factors affecting lipid homeostasis in health and in IR state

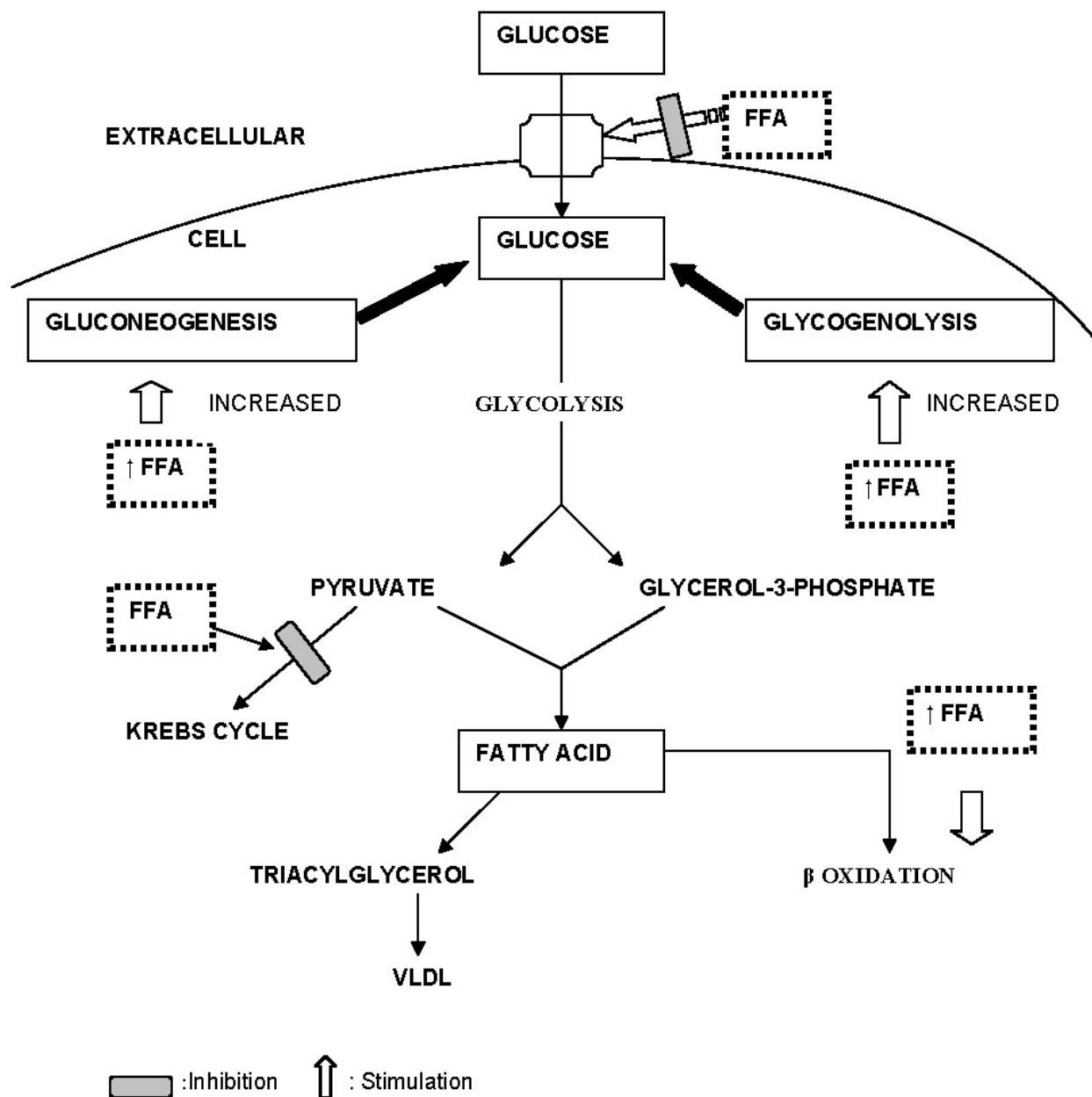
CYTOKINES/HORMONES	HEALTH		IR STATE
INSULIN	↑ Lipogenesis	↓ Lipolysis	↑ Lipogenesis & Lipolysis
LEPTIN	↓ Lipogenesis	:	↑ Hepatic Lipid Turnover
TNF	↓ Lipogenesis	↑ Lipolysis	↑ Hepatic inflammation
ADIPONECTIN	↓ Lipogenesis	↓ Lipolysis	↓ Levels correlate with increased hepatic damage
IL-6	↓ Lipogenesis	↔	↑ Lipogenesis, ↓ Hepatic lipid Turnover

both genetic and environmental factors. Inherited disorders of IR are extremely rare and include leprechaunism, Rabson-Mendenhall syndrome and type A insulin resistance syndromes (56). The genetics of IR and the phenotypic variations that may be observed as a consequence of isolated gene defects have been studied in detail using single gene knockout mouse models (Table 1). While these models are excellent tools for understanding the specific mechanisms of IR, they do not reflect the genomic alterations of the actual disease process in the body. The reason for this difficulty is due to the polygenic nature of IR and the polymorphism of different genes involved in the process of pancreatic insulin secretion and their action on specific target organs.

One of the key targets of insulin action both in health and in disease is the adipose tissue. From the evolutionary perspective, adipose tissues were developed as a source of stored energy to be used during times of metabolic stress (adolescence, pregnancy, wound healing) and limited food availability. Hence the body was adapted to store excess caloric intake during the times of relative abundance as fat in the adipose tissues. Insulin, therefore served as a sensor to detect the overall metabolic status of the body and direct the cellular apparatus towards lipogenesis in times when excess energy was available. However with the change in dietary habits and activity

scale as seen in present times, the predominantly adaptive response to deal with metabolic stress has been altered to a pathological state with failure of homeostatic regulation of cellular metabolism. The adipose tissue as a consequence serves as the major site of insulin action in both metabolic syndrome and NAFLD. The balance between lipid storage and mobilization is determined by genetic factors, location of adipose tissue and neurohumoral pathways e.g.  $\beta_3$  adrenergic neurons (57). Insulin is the principal agent involved in lipogenesis while the counterregulatory hormones such as glucagon, growth hormone and epinephrine control lipolysis. In addition to these hormonal factors, adipocytokines such as leptin, TNF, adiponectin produced locally by the adipocytes are also involved in the process of modulating lipid metabolism (58-60) (Table 2). In the IR state, the energy balance of the cell shifts towards lipid mobilization due to the effects of aberrant insulin action in concert with the actions of pro-inflammatory cytokines and excess FFA.

One of the principal ways that glucose is cleared from the circulation is by uptake into the striated muscles. In IR state this ability of striated muscle to handle glucose load is impaired. This is primarily due to decreased translocation of GLUT-4 to the cell surface resulting in a decreased rate of internalization of glucose into the cell. The specific mechanisms for abnormalities of GLUT-4 translocation



**Figure 4.** Effect of free fatty acids on intermediary metabolism in insulin resistant state.

in the process of IR remains largely unknown. It is however well established that FFA impairs insulin signaling, and the degree of fat deposition in the skeletal muscles show a good correlation with degree of IR (61). Therefore it can be said that impairment of both insulin signaling and glucose uptake at the level of striated muscles contributes to the process of insulin resistance and leads to impaired glucose homeostasis.

## 5.2. Metabolic consequence of insulin resistance

Insulin serves as the common regulatory link between carbohydrate and fat metabolic pathways in the body. Thus it is hardly surprising that both glucose and fat homeostatic process are affected as a consequence of

insulin resistance. Obesity with its inherent increased adipose tissue mass increases the FFA delivery to the liver. This FFA both at the level of the adipose tissues and the liver impairs insulin signaling and promotes hyperinsulinemia and IR. IR in turn results in impairment of normal insulin induced suppression of peripheral lipolysis resulting in increased FFA levels. This leads to a vicious cycle in which increased FFA and IR creates a positive feedback loop resulting in disordered glucose and fat homeostasis (Figure 4). The sensitivity of adipocytes to insulin has been studied experimentally by looking at suppression of FFA levels by circulating insulin levels (62). It has been seen that the FFA which are markers of

peripheral lipolysis are half maximally suppressed at insulin concentrations of 20:U/mL (63). This data shows that adipose tissues are extremely sensitive to the actions of insulin and small changes in insulin concentration bring about a marked alteration in the rate of peripheral lipolysis. The net result of increased peripheral lipolysis in the IR state causes increased hepatic lipid handling and generation of oxidative stress. Also the impairment of hepatic insulin signaling results in the inhibition of the krebs cycle and the stimulation of gluconeogenesis. This results in increased hepatic glucose output. Visceral adipocytes are more susceptible to fat mobilization when there is failure of insulin mediated suppression of lipolysis. This is most likely a consequence of 11 $\beta$  (OH) dehydrogenase over-expression in these tissues (64). In addition to the inherent genomic aberrations that are observed in the adipose tissues, pro-inflammatory adipocytokines released from these sites also interfere with insulin signaling and contribute to the genesis of IR state.

Glucose homeostasis at the periphery is regulated to a large extent by the insulin induced glucose uptake by the striated muscle. Almost 80% of the circulating glucose is normally taken up by the skeletal muscle, a process that is impaired in IR state (65). As a result of the decreased uptake of glucose, there is a compensatory increase in pancreatic insulin secretion. This compensatory increase fails over time and overt diabetes develops. Thus the relationship between plasma insulin levels and fasting glucose levels have an inverted horseshoe shape over the natural course of the IR syndrome. Another important contributor to the process of insulin resistance at the periphery is the accumulated FFA in the skeletal muscles. Consequently, there is inhibition of serine phosphorylation of insulin receptor substrate (IRS-1) and aberrant insulin signaling cascade (66). Additional support for these observations has been obtained from experimental data showing that short term infusion of lipid emulsions can induce profound insulin resistance (67, 68). Again the result of the impairment of insulin signaling due to lipotoxicity at the level of skeletal muscles eventually causes increased hepatic glucose output. These data support a close and integral link between glucose and lipid metabolic pathways. This integration of glucose and fat metabolism at the visceral adipose storage sites as a result of lipolysis and efflux of FFA is known as the “single gateway hypothesis” (69).

In obese individuals, IR is present both at the periphery as well as at the level of the liver. However some degree of hepatic insulin sensitivity is still maintained but the extent to which this sensitivity contributes to lipogenesis is largely unknown. The molecular mechanics of insulin induced lipogenesis involves inhibition of protein kinase A (PKA) and activation of PEPCK. The net consequence of these enzymatic alterations is inhibition of lipoprotein lipase and increased formation of acetyl CoA. Acetyl CoA serves as a common starting point for both cholesterol and fatty acid synthesis. Thus both the lipogenic and the cholesterol synthetic pathway are affected in IR state. Support for the role of insulin in the lipogenic process has been obtained from data showing increased postabsorptive hepatic lipogenesis in obese

individuals (70). This increased hepatic lipogenesis was not accompanied by increase in peripheral adipose tissue lipogenic capacity. It is however well established that visceral adipose tissue and not subcutaneous adipose tissue is correlated with IR state which in turn tends to suggest an important role for the local milieu of target organs in modulating its response. While the contribution of this increased hepatic lipid production would be small on a day to day basis, over the long term it would be cumulative and could contribute to the pathobiology of obesity (Figure 2).

### 5.3. IR and genesis of NAFLD

The common metabolic abnormality that is observed across the entire spectrum of fatty liver disease is IR. While the contribution of IR in the pathogenesis of fatty liver has been well established, its role in the genesis of steatohepatitis has yet to be proven.

### 5.4. Fatty liver

The development of fatty liver is dependent on the disruption of the balance between lipogenesis and lipolysis that is seen normally in healthy individuals. Lipid homeostasis is normally closely linked with glucose homeostasis and reflects the overall caloric state of the body. In health, increase in insulin levels in the post absorptive state serves to divert excess lipids to either storage as VLDL or into oxidative pathways for generation of ketone bodies depending on energy status of the individual. However in obesity and metabolic syndrome, this adaptive response of the body is disrupted and the entire hepatic machinery gets directed towards diverting the FFA into TG formation as well overwhelming the mitochondrial and peroxisomal lipid oxidation pathways. The evidence supporting the role of hyperinsulinemia in the process of development of fatty liver has come from several sources. Clinically it has been observed that patients on peritoneal dialysis who have insulin added to their dialysate have a thin rim of steatosis developing around their livers (71). Also focal hepatic steatosis has been attributed to preferential drainage of blood with high insulin concentrations into the liver by the branches of the portal vein. There is also a strong correlation between the degree of hyperinsulinemia and the extent of hepatic steatosis (72). At the nuclear level it has been shown that insulin brings about changes responsible for lipogenesis by virtue of its action on SREBP-1c and FAS genes (73)(Table 3). The upregulation of these key lipogenic genes may explain the inability of caloric restrictions to decrease post absorptive hepatic lipogenesis, at least over a short period of time (70). All these observations tend to suggest that insulin is the primary pathologic stimulus towards development of fatty liver, and an abnormal signal transduction cascade is central to the genesis of aberrant insulin action at the level of target organs.

Although the lipid handling in the liver is abnormal in both fatty liver and NASH, no systemic defects in fat metabolism have been detected in subjects with these disorders (17). However defects in the incorporation of leucine into apolipoprotein B-100 have been described in patients with NASH (74). The presence of such a defect in the context of increased hepatic lipid flux increases the likelihood of hepatic lipid handling machinery switching from



**Table 3.** Insulin regulation of genes involved in intermediary metabolism

FAT METABOLISM		CARBOHYDRATE METABOLISM	
<i>Activated by Insulin</i>	<i>Inhibited by Insulin</i>	<i>Activated by Insulin</i>	<i>Inhibited by Insulin</i>
SREBP 1c	FKHR	GK	PEPCK
FAS	APO CIII	PK	GSK 3
ACC	HSL	PFK 1	Glc-6-Pase
LXR	CPT-1		F1,6-Pase
SCD			

SREBP: sterol regulatory element binding protein, FAS: fatty acid synthetase, ACC: acyl CoA carboxylase, LXR: Liver X receptor, SCD: steroyl CoA desaturase, FKHR: forkhead related protein, APO CIII: apolipoprotein CIII, HSL: hormone sensitive lipase, CPT: carnitine palmitoyl transferase, GK: glucokinase, PK: pyruvate kinase, PFK: phosphofructokinase, PEPCK: phosphoenolpyruvate carboxykinase, GSK 3: glycogen synthetase kinase 3, Glc-6-pase: glucose 6 phosphatase, F1,6-pase: fructose 1,6 phosphatase

a state of lipid oxidation to lipid accumulation. At the molecular level, it has been shown that overexpression of suppressors of cytokine signaling (SOCS 1&3) in the liver induces insulin resistance and increase in SREBP 1c gene expression (75). The predominant mechanism behind these effects are SOCS mediated antagonism of STAT 3 inhibition of SREBP 1c promoter activity. These data tend to suggest that aberrant regulation of cytokine activity may initiate IR and increased hepatic lipogenesis. Other studies looking at animal models with hepatic over expression of 11(OH) dehydrogenase have found a strong correlation with IR and hepatic steatosis in the absence of obesity (76). While the data obtained from these animal studies cannot be generalized to reflect the molecular mechanisms operating in human fatty liver disease, they do clarify our understanding of the link between hyperinsulinemia and hepatic lipogenesis.

## 6. NASH

The natural history of NAFLD involves progression from steatosis to steatohepatitis and finally cirrhosis. While the pathologic process involved in the development of cirrhosis is similar to other chronic liver diseases, the same cannot be said about steatohepatitis. It is still unclear why despite having hyperinsulinemia and IR as a common link, some patients develop necroinflammatory activity characteristic of NASH while others continue to have simple steatosis. It is however clear that a second insult is necessary on the top of steatosis for steatohepatitis to develop. The second insult or "hit" develops when the redox state of the cell is altered and oxidative stress develops. At the cellular level oxidative stress results from the generation of reactive oxygen species (ROS) or depletion of antioxidant defenses. Therefore at any given point of time the balance between the pro and antioxidant forces within the cell determines the phenotypic presentation of steatohepatitis versus simple steatosis.

Experimental evidence of oxidative stress has been observed in steatosis and steatohepatitis. Elevated 3-nitrotyrosine levels have been seen in patients with fatty liver with the levels increasing when steatohepatitis develops (17). This may represent a threshold limit for oxidative stress before necroinflammatory activity is initiated or simply a matter of biological variability in

terms of individual susceptibility to oxidative stress.

The genesis of oxidative stress depends on the production of reactive oxygen species. In the liver the primary sites for generation of ROS include the mitochondria, peroxisomes, cytochrome P-450, NADPH oxidase, cyclo-oxygenase and the lipo-oxygenase system (77-80). The mitochondrial, peroxisomal and the cytochrome systems are the major contributors to the process of oxidative damage with other components having a lesser role.

In the face of increased FFA load and IR, the hepatic lipid handling machinery diverts most of the FFA into mitochondrial oxidative chain. Animal studies have indicated that this increased oxidation may be a result of continued CPT-1 activation as a result of decreased sensitivity to feedback inhibition by malonyl CoA (81). Similar mechanisms could explain the continued FFA oxidation in the face of increased insulin levels that normally would inhibit CPT activity and mitochondrial entry of FFA. Functionally, there is increased cycling of FFA across the inner mitochondrial membrane leading to re-entry of a proton into the matrix during each cycle. This proton cycling bypasses the key enzyme ATP synthetase resulting in decreased ATP formation and dissipation of energy as heat. Along with this decreased ATP formation and regeneration, increased oxidation results in the formation of reactive oxygen species (ROS). The formation of ROS results in lipid peroxidation, the byproducts of which initiates the process of oxidative stress. There is damage to the mitochondrial DNA and alteration of mitochondrial proteins including cytochrome-c oxidase and adenine nucleotide translocator. These alterations impair electron movement along the respiratory chain and impair further production of ROS that leads to more lipid peroxidation. Eventually the cytochrome depletion overwhelms the intrinsic compensatory capacity of the mitochondria leading to apoptosis and cell death. An added dimension to the process of ROS mediated cell damage is provided by TNF- that is released from adipocytes and Kupffers cells in response to the oxidative stress. TNF- impairs mitochondrial electron transfer chain and opens up mitochondrial permeability transition pores resulting in depletion of cytochrome oxidase (82, 83). In the face of oxidative stress, patients with NASH also show mitochondrial ultra-structural changes manifested by

formation of megamitochondria and paracrystalline inclusion bodies (17).

The cytochrome P-450 system is also involved in the genesis of mitochondrial oxidative stress. Two of the key inducible hepatic microsomal enzymes in this process include the CYP2E1 and CYP4A, both of which are capable of initiating and perpetuating lipid peroxidation. It has been shown that CYP2E1 activity is not only increased in patients with NASH but also in those with features of metabolic syndrome and steatosis in the absence of steatohepatitis (84). This is most likely due to the loss of repressive effect of insulin in the IR state, which is a common theme in all these disorders. The roles of CYP2E1 and CYP4A appear to be complementary in the process of lipid peroxidation. However, CYP2E1 has been more extensively studied in NASH with most evidence suggesting increased activity in the acinar zone 3, the site of maximal damage in this condition (77, 85). Increased expression of CYP2E1 results in NADPH dependent lipid peroxidation due to formation of oxyradicals and generation of oxidative stress. Clinically patients with NASH have increased levels of  $\alpha$ -(OH) butyrate levels as a result of increased oxidation that have been shown to correspond with overexpression of CYP2E1 (86). However in contrast to increase in CYP2E1 expression seen in response to alcohol in alcoholic liver disease that abates as soon as alcohol is withdrawn, in NASH the over-expression persists and continues to propagate lipid peroxidation. Hyperinsulinemia induced CYP2E1 overexpression in NASH, is most likely secondary to posttranslational enzyme stabilization although more direct transcriptional control also may have a role in this process (87-90).

The role of PPAR activation in the pathogenesis of NASH has also been studied in both animal models of steatohepatitis and human subjects. It has been shown that in transgenic mouse models with absence of fatty acyl CoA oxidase (AOX), there is accumulation of long chain fatty acids leading to mitochondrial damage and florid steatohepatitis (91). This is thought to be due to the induction of CYP4A by PPAR- activation by the accumulated LC CoA. Furthermore PPAR- and AOX null mice do not develop mitochondrial injury or steatohepatitis in the face of excess lipid peroxidation (92). Animal studies have also shown improvement of glucose tolerance and insulin sensitivity following activation of PPAR $\alpha$  receptors (93). Evidence from these animal studies is supported by human data showing the improvement in hepatic steatosis and IR following stimulation of PPAR $\alpha$  receptors (94). Taken together, these findings from human and animal studies support an important role played by the PPAR system in modulating the severity of steatosis and steatohepatitis in response to excessive hepatic lipid handling.

The effect of pro-inflammatory cytokines released from the hepatic stellate cells (HSC) in response to lipid peroxidation has also been studied. TNF $\alpha$  has been shown to activate HSC and stimulate production of TGF $\beta$ , a potent stimulus for fibrogenesis. It also induces the up-regulation of NF- $\kappa$ B, a key transcriptional mediator for

development of necroinflammatory changes associated with steatohepatitis (95). There is also evidence showing that CYP2E1 dependent oxidative stress is associated with increased collagen I gene expression, suggesting a direct role for ROS in stellate cell activation (96).

Another potential mechanism for damage in steatohepatitis that has been a subject of investigation for some time now is the depletion of protective endogenous anti-oxidants. The depletion of anti-oxidants and vitamins such as vitamin E during the process of lipid peroxidation has been described in experimental steatohepatitis (53). The main roles of these anti-oxidants are free radical scavenging and inactivation of ROS. Studies have described depletion of antioxidant paraoxonase-1 in insulin resistant states with the degree of depletion corresponding with the severity of metabolic syndrome (97). However, from the therapeutic standpoint the promise of these experimental evidences has not been fulfilled in human studies. While the levels of antioxidants such as Vit E have been found to be lower in patients with metabolic syndrome, supplementation of Vit E has failed to prevent hepatic damage in patients with NASH (98).

## 7. CONCLUSION

In conclusion, there is ample evidence now to suggest a cause and effect relationship between IR and NAFLD. As work continues, it has become quite evident that there are multiple abnormalities involving both the lipid and carbohydrate metabolism in the pathogenesis of NAFLD. Overall there is a failure of endogenous autoregulation with excessive FFA overwhelming the hepatic lipid handling machinery. Hepatic lipotoxicity leads to aberrant insulin signaling and resultant hyperinsulinemia. The phenotypic response to these maladaptive changes is steatosis and in some cases steatohepatitis. Thus insulin plays a key role in the metabolic integration of both lipid and carbohydrate handling pathways and is central to the pathogenesis of NAFLD.

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