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Review

Insights into the pathogenesis of NAFLD: The role of metabolic and pro-inflammatory mediators.

E. Xirouchakis[1], Penelopi Manousou[2], Lemonia Tsartsali[3], S. Georgopoulos[1], A.K. Burroughs[2]

SUMMARY

Non alcoholic fatty liver disease (NAFLD) is a part of the metabolic syndrome which also comprises obesity, dyslipidaemia, type 2 diabetes mellitus and hypertension. The importance of NAFLD is due to its high prevalence and its potential to evolve into cirrhosis and hepatocellular carcinoma. In recent years several molecular pathways have been shown to be involved in this syndrome. Moreover the role of many cytokines has been elucidated. In the present review we discuss the recent evidence regarding the role of metabolic and proinflammatory mediators in the pathogenesis of NAFLD.

Key words: Non alcoholic fatty liver disease, cytokines, insulin receptor, leptin receptor, adiponectin.

INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) comprises simple steatosis of the liver, steatohepatitis and cirrhosis.[1] It is considered as part of the metabolic syndrome, which affects adults and children, commonly associated with visceral adiposity, dyslipidaemia, hyperglycaemia and hypertension. Its importance is due to its high prevalence of up to 31% in the general population,[2] and its ability to evolve in cirrhosis, hepatocellular carcinoma, and liver failure.[3]

[1]GI and Hepatology Department, Athens Medical, P. Falirou Hospital, Athens, Greece, [2]The Sheila Sherlock Hepatobiliary Pancreatic and Liver Transplantation Unit, Royal Free Hospital, London, UK, [3] First Pediatric Department "Agia Sofia" Children's Hospital, Athens, Greece.

Author for correspondence:

Elias Xirouchakis MD, Correspondence Address: GI and Hepatology Department, Athens Medical, P. Falirou Hospital, 36 Areos, 175 62, Athens, Greece, Tel: 0030 2109212662, e-mail: elmoxir@yahoo.gr

Studies in obese patients who had bariatric surgery showed cirrhosis on average in 5.8% (range 1.6% to 10%).[2]

Recent evidence on the etiology of the disease indicates multifactorial causes.[2-4] Studies in animal models and humans indicate that both its presentation and its course are influenced by genetic and environmental factors [2;4]. Altered signaling pathways and an aquired cytokine profile are becoming important pathogenetic factors.[5-7] The present review focuses on the role of receptors and cytokines in the pathogenesis of NAFLD.

ETIOLOGY OF NAFLD

According to the currently accepted 'two-hit' theory [4;8] simple liver steatosis, results from an imbalance between hepatic fatty acid synthesis, uptake, oxidation and export (the first hit) whereas the following second hits like oxidative stress, pro-inflammatory cytokine release, lipopolysaccharide and ischemia-reperfusion injury favor the development of steatohepatitis. The main histologic feature in simple steatosis is the presence of fat containing vacuoles (vescicles) within the liver cells, whereas steatohepatitis which is considered a more aggressive form is characterised histologically by the presence of inflammatory cells, hepatic cell necrosis, and fibrosis.

The adipose tissue cells, called adipocytes are adapted to store large quantities of fatty acids (FA) as triglycerides (TG) and to release them as a fuel on demand. An acquired adipocytokine profile during obesity influences free FA (FFA) release from adipocytes with the consequent enhancement of lipid delivery to the liver.[9] In fact adipocytes, secrete and influence actions of a variety of cytokines, including adiponectin, leptin, tissue factor, angiotensinogen, lipoprotein lipase, interleukin-6, plasminogen activating factor-1 and others.[10]

Under normal conditions in liver cells lipids do

not accumulate but rather are transformed into mixed particles like Very Low Density Lipoproteins (VLDL) which then can be secreted in the blood stream. The first event in the development of NAFLD is the accumulation of circulating FFA in the liver. Fatty acids enter into liver cells by simple diffusion and accumulate in high amounts (macrovescicular steatosis).[11] Whenever a large amount of fatty acids is present in the liver environment, liver cells increase lipid degradation pathways (lipolysis) but also suppress other pathways including insulin receptor activation.[7] Dysfunction of the insulin receptor causes hyperglycemia and secondary hyperinsulinaemia which are common features in the metabolic syndrome. Another important feature of these metabolic alterations is increased de novo lipogenesis which is observed in NAFLD patients with hepatic insulin resistance, activated by the presence of glucose and hyperinsulinaemia,[12] and by low-grade inflammation and increased tumor necrosis factor (TNF). While in normal subjects, the contribution of hepatic de novo lipogenesis to the pool of hepatic fatty acids is less than 5%, it increases up to 25% in NAFLD patients.[9]

Another very important step in the pathogenesis of NAFLD is oxidative stress. Hyperglycemia can induce oxidative stress by several different mechanisms. Autoxidation of glucose and the nonenzymatic glycation of proteins generate free oxygen radicals.[13] Glucose reacts slowly (non-enzymatically) with free amino groups of proteins to form glycation products. Glycation products are further degraded to advanced glycation end-products (AGEs), a process accelerated by oxidative stress.[13] Several reports show that AGEs have an important impact on cytokine release leading to an inflammatory reaction.[14] Moreover oxidative stress in the intracellular environment produces damage of membrane-bound organelles such as mitochondria, lysosomes and endoplasmic reticulum, causing mitochondrial dysfunction, activation of lysosomes, endoplasmic reticulum stress and finally hepatocyte apoptosis.[15-17]

Thus lipid accumulation in non adipose tissues and the relative cytokine profile acquired, has a major role in the development of an altered metabolic state causing important changes in targeted organs like the liver [6;7]. In the following sections we will explore in more detail all those metabolic changes that have been described to play a role in the pathogenesis of NAFLD.

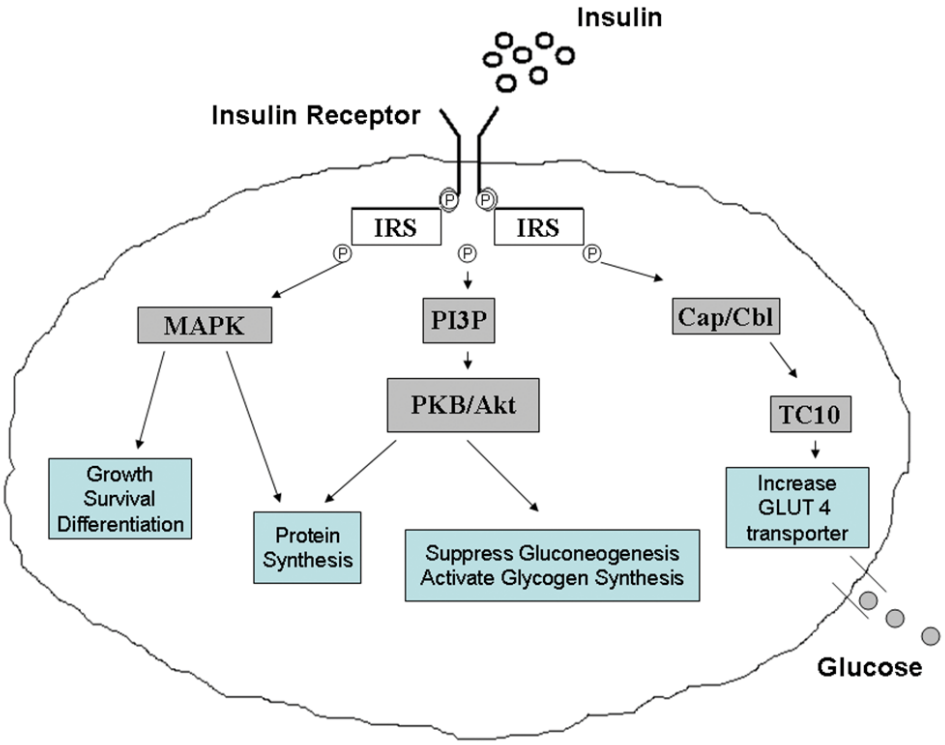


Figure 1. Intracellular insulin signalling pathways. Phosphorylation of insulin receptor substrates (IRS) by activation of insulin receptor is the first step before the activation of the three main intracellular pathways, the phosphatidylinositol 3-Kinase (PI3K)/protein kinase B (PKB)/Akt pathway, the mitogen-activated protein kinase (MAPK) pathway, and the CAP/Cbl/Tc10 pathway.

THE ROLE OF INSULIN AND ITS RECEPTOR

Several studies have elucidated the importance of insulin resistance in the initiation and development of NAFLD.[17-19] The insulin receptor which is found in hepatocytes, pancreatic cells, muscle cells and endothelial cells seems to be the one involved in the pathogenesis of NAFLD.[7] It belongs to a subfamily of receptor tyrosine kinases that includes insulin-like growth factor-1 and insulin receptor- related receptor (Figure 1).[20]

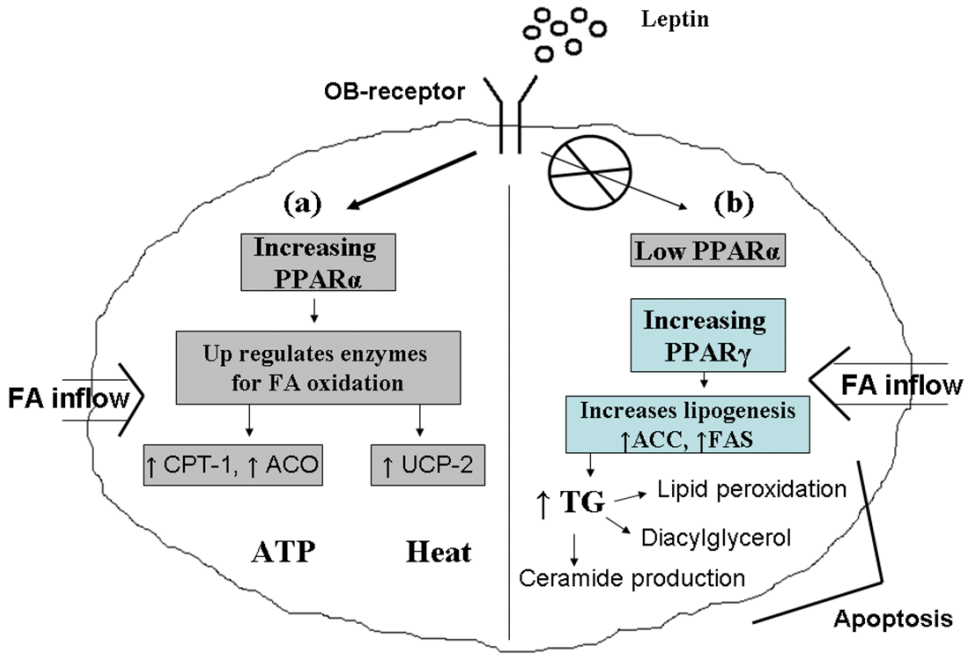
Dysfunction of the insulin receptor results in alterations of glucose homeostasis. Hepatic insulin resistance refers to impaired suppression by insulin of glucose production by hepatocytes.[21] Failure of insulin to inhibit hepatic gluconeogenesis and glycogenolysis is to a large extent responsible for the development of fasting hyperglycaemia and persistent stimulation of insulin production by pancreatic beta cells.[22,23] Recent evidence showed that hepatic insulin resistance is more important than peripheral insulin resistance in order to develop hyperglycaemia and glucose intolerance. Animals with tissue specific deletion of the insulin receptor have been invaluable to dissect single components of insulin signaling and to demonstrate the importance of the liver for insulin resistance.[24,25] Mice lacking insulin receptors in hepatocytes exhibit dramatic insulin resistance, severe glucose intolerance and failure of insulin to regulate hepatic gene expression and to suppress hepatic glucose output.[26] In contrast, normal glucose and insulin levels are found in mice with a deletion of insulin receptor in skeletal muscle.[27,28] Deletion of insulin receptor in adipose tissue is associated with low insulin levels suggesting improved insulin sensitivity.[29] When insulin receptor is simultaneously knocked out in fat and muscle, there is no change in glucose or insulin levels.

At the molecular level several studies have clarified the involvement of specific intracellular pathways in the pathogenesis of insulin resistance. Under normal circumstances the PI3K/PKB/Akt pathway is involved in controling metabolic actions by insulin, transcription of glucose transporter GLUT-4, protein synthesis via the mammalian target of rapamycin mediator, mTOR,[24] and control of cell survival. The activation of this pathway mediates glycogen synthesis, via PKB/Akt inhibitory phosphorylation of glycogen synthase kinase 3 (GSK3), a kinase that negatively regulates glycogen synthase. It also inhibits, via PKB/Akt-activation of the forkhead transcription factor (FOXO-1), the transcription of key enzymes for gluconeogenesis: phosphoenolpyruvate carboxy-kinase (PEPCK) and glucose-6-phosphatase. Thus through activation of PI3K and PKB/Akt, and subsequent inactivation of GSK3 and activation of FOXO-1, insulin promotes storage of glucose as glycogen and inhibits glucose synthesis and glucose output. The MAPK pathway, influences protein synthesis and the mitogenic, growth and cell differentiation effects on hepatocytes. Activation of this pathway favors transcription of an important factor, the sterol regulatory element binding protein (SREBP-1c) which mediates most of insulin's effects on lipogenesis, by regulating all of mono-unsaturated fatty acids synthesis.[30] The CAP/Cbl/Tc10 pathway controls the membrane translocation of glucose transporter-4 (GLUT4). Insulin uses the latter pathway to regulate glucose intake in GLUT4-expressing cells like adipocytes and muscle cells. However in hepatocytes glucose transport is mediated through the GLUT2 transporter. With insulin resistance, in hepatocytes not all of the above mentioned insulin signaling pathways are affected the same. It seems that the PI3K/PKB/Akt pathway is the one more severely affected, leading to loss of control over glucose output, causing hyperglycaemia and compensatory hyperinsulinaemia.[7]

In conclusion insulin resistance is due to an altered function of the insulin receptor. Insulin resistance can be acquired through multiple mechanisms, and may affect various steps in the insulin signaling cascade which finally suppresses important metabolic pathways causing overproduction of glucose through reduced gluconeogenesis and increased glycogenolysis. Although insulin participates in the control of cell cycle and cell survival pathways in hepatocytes, the effects of intrahepatic insulin resistance on proliferation and apoptosis remain to be explored.

THE ROLE OF LEPTIN AND ITS RECEPTOR

Leptin is a 16kDa peptide hormone with complex metabolic actions released by adipose tissue in a mass-dependent manner. Leptin receptor (OBR) (Figure 2) is structurally similar to a class I cytokine receptor.[31] On binding leptin, the receptors form tetrameric complexes (two receptors with two molecules of leptin) and the intracellular domain undergoes ligand-induced conformational change. Several alternative splice variants with a single transmembrane domain and a cytoplasmic region of variable length have been described.[32] The short OBRa isoform has not been shown to have any signalling activity. In contrast, the OBRb isoform has a long intracytoplasmic region that contains signal transduction motifs that activate the JAK/STAT protein kinase signal transduction cascade.[33] In physiological states, leptin mediates an innate adaptive neuroendocrine response to starvation. As adipose tissue is depleted, leptin levels fall. This increases



Footnote: carnitine palmitoyl transferase 1 (CPT-1), acetyl CoA oxidase (ACO), uncoupling protein-2 (UCP-2), acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS), triglycerids (TG), Fatty acids (FA), peroxisome proliferator-activated receptor (PPAR).

Figure 2. Management of caloric excess in non adipose tissues. When increased amounts of fatty acids flow into cells: (a) with an intact leptin system PPARα activation induces lipolysis with production of energy. (b) when leptin levels or its receptor become deficient PPARγ activation leads to increased lipogenesis.

the desire to seek food and suppresses the thyroid, growth hormone, adrenal and reproductive endocrine pathways.[34] By acting on the hypothalamic ventral median nucleus receptors it inhibits release of neuropeptide Y (NPY), the most powerful mediator of eating and drinking.[35] The absence of hypothalamic leptin action results in elevated NPY levels[36] and a marked hyperphagia.[37] Although leptin can alter hypothalamic functions it does not prevent lipid accumulation in the adipocytes[38] and most normal humans and rodents become obese when offered a high-fat/high-carbohydrate diet despite plasma leptin levels that rise.[39] However evidence suggests that the presence of high leptin levels during obesity prevents the accumulation of lipids in non adipose tissues[40-42] and protects from fatty acid overload. Whenever the FA supply in non adipose tissues exceeds their oxidative needs, the transcription factor PPARα promotes compensatory oxidation of the surplus FA by upregulating enzymes of FA oxidation (CPT-1 and ACO) (Figure 2). The unneeded energy which is produced will be dissipated as heat, thanks to upregulation of uncoupling protein (UCP)-2. This compensatory system requires the presence of leptin and a normal leptin receptor (OBR).

Rodents that lack leptin action whether as a consequence of aleptinemia or of leptin resistance, develop hyperphagia and obesity. Thus when leptin becomes deficient or non functioning, due to unresponsiveness of its receptor, fatty acids accumulate in non adipose tissues.[6] The expression of PPARα is reduced and of another transcription factor PPARγ, increased (Figure 2). The surplus of FA influx is considered to bind to the latter, which then increases transcription of lipogenic enzymes. This causes increased nonoxidative FA metabolism and apoptosis. Thus high fatty acid disposal in cells due to leptin dysfunction is the main reason for steatosis, but there is evidence that de novo fatty acid synthesis from glucose also occurs. This is shown by overexpression of crucial lipogenic transcription factors, like sterol regulatory element binding protein (SREBP-1c)[43] and peroxisome proliferator-activated receptor (PPAR-γ), and their lipogenic target enzymes, acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS), liver-type fatty acyl CoA synthase (L-ACS), and glycerol phosphate acyl transferase (GPAT).[44]

The lipid excess observed in the pancreatic islets of ZDF (*fa*/*fa*) rats is largely the result of increased lipogenesis, although decreased FA oxidation also contributes.[44,45]

When normal pancreatic islet cells are exposed to a surplus of FA, FA oxidation rises above the actual oxidative needs of the cell. This compensatory oxidation is probably triggered by the FA themselves, which serve as a ligand for PPARγ[46-48] and thereby increase expression of its target enzymes of FA oxidation, carnitine palmitoyl transferase 1 (CPT-1) and acetyl CoA oxidase (ACO).[49] Thus, the oxidative machinery for disposal of the excess is upregulated. Recently, however, the key role of the lipogenic enzyme, ACC-2, which catalyzes the production of malonyl-CoA in FA oxidation, has become increasingly apparent.[50] Malonyl CoA can inhibit long-chain FA oxidation by suppressing CPT-1 activity.[51] Thus, deletion[50] or inhibition of ACC-2[52] will increase CPT-1 activity, by reducing malonyl CoA and raises the rate of FA oxidation. Leptin lowers ACC mRNA in association with increased expression of uncoupling protein-2 (UCP-2).[53] This implies that the leptin-dependent compensatory oxidation generates heat rather than ATP. Failure of FA to suppress appropriately the high levels of lipogenic enzymes, ACC and FAS, results in a progressive intracellular accumulation of FA and their nonoxidative metabolites. This seriously compromises cellular function, leading to apoptosis.

When compensatory oxidation of excess FA fails, the FA surplus must ultimately enter pathways of nonoxidative metabolism. Initially, TG appear to be the major lipid product, but they probably do little to the cell. Ultimately, however, hydrolysis of the TG stores will increase the already expanded FA pool, providing additional substrate for nonoxidative FA metabolism.

When leptin action is lacking, the enzyme serine palmitoyl transferase (SPT)[54] is expressed at high levels,[55] thereby increasing the condensation of palmitoyl CoA and serine to form dihydrosphingosine, the first step in de novo ceramide biosynthesis. Apoptosis is believed to result from excessive ceramide formation coupled with underexpression of the antiapoptotic factor, Bcl2.[56,57]

Thus dysfunction in the production of leptin or its receptor, affect the metabolic pathway of the triglycerides with PPAR-α down-regulation being the link between the abnormalities observed in the visceral adipose tissue, and the hepatic features of NASH.[58] This indicates that reduced lipid breakdown occurs in addition to increased fatty acids afflux to the liver, with consequent activation of different metabolic pathways like ceramide biosynthesis with more deleterious effects on cells.

THE ROLE OF ADIPONECTIN

Adiponectin is a protein exclusively secreted from adipose tissue, able to increase insulin sensitivity and free FA oxidation in the liver and to exert anti-inflammatory effects.[59] It circulates in the bloodstream as multimere of full-length proteins or as cleaved proteins containing only the globular domain.[60] Although the three-dimensional structure of adiponectin closely resembles that of TNF-α, these two proteins have completely opposite effects. Both *in vivo* and *in vitro* experiments demonstrate that adiponectin and TNF-α antagonize each other's action in their target tissues.[61] Adiponectin exerts its effects by binding to its receptors. Hepatocytes express mainly adipoR2, which binds with equal affinity to the full-length adiponectin and to the cleaved globular form. Upon adiponectin binding, the receptor activates the transcription factor peroxisome proliferator-activated receptor (PPARα) and stimulates the activity of AMP-dependent kinase (AMPK).[62] Activation of PPARα enhances transcription of the enzymes of the fatty acid b-oxidation machinery[63] and has anti-inflammatory consequences, probably through transrepression of nuclear factor-kB (NF-kB).[61] The activation of AMPK, via regulation of acyl CoA-carboxylase activity and intracellular malonyl-CoA concentrations, inhibits de novo lipogenesis and favours fatty acid b-oxidation.[64] Thus, adiponectin opposes intrahepatic lipid accumulation. This mechanism is largely implicated in the insulin sensitising effect of adiponectin. In NAFLD patients, serum adiponectin is negatively correlated with hepatic insulin resistance and to the amount of fat in the liver.[65,66]

THE ROLE OF PRO-INFLAMMATORY CYTOKINES

Several proinflammatory cytokines may be pathogenetic in NAFLD. In obese and/or diabetic subjects increased serum levels of TNF and IL-6 originate largely from adipose tissue. In the liver, Kupffer cells are capable of releasing, among other factors, large quantities of TNF and IL-6 directly in contact with liver cells influencing hepatic inflammation and fibrogenesis.[67]

In the adipose tissue, TNF acts by repressing genes involved in uptake and storage of non-esterified fatty acids.[4] These fatty acids are thus readily available to accumulate in non adipose tissues like the liver, increasing the pool of intrahepatic FFA. Increased levels of FFA can also induce TNF-a expression within the hepatocytes[68] (Figure 3).

In the liver TNF activates stress-activated protein kinase (JNK1), and inhibitory kappa B kinase beta (IKKb). JNK activation further induces TNF, forming an autocrine/paracrine loop which potentiates insulin resistance.[69,70] IKKb activation causes phosphorilation of the IKB pro-

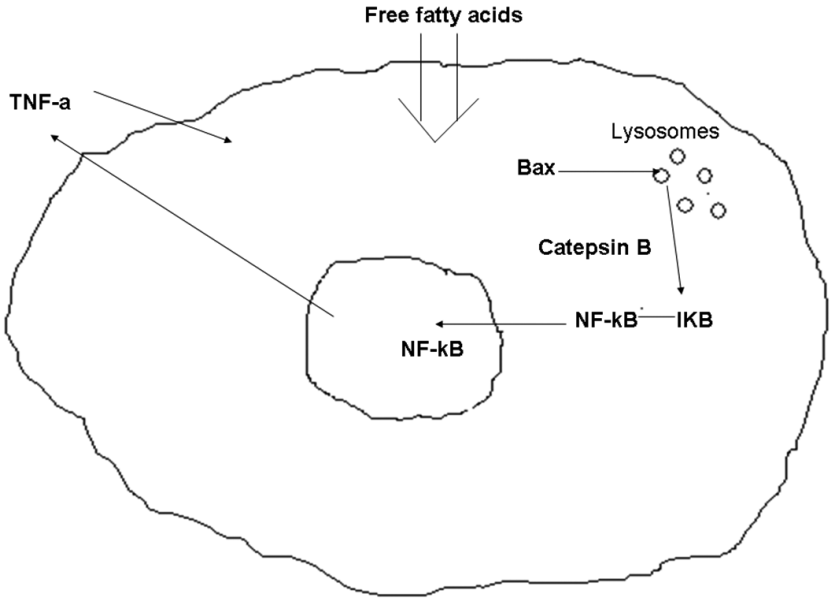


Figure 3. When free fatty acids enter the liver cells are causing translocation of a protein called Bax from the cytosol to the lysosomes. This induces lysosome membrane permeabilization and catepsin B redistribution into the cytosol. Once in the cytosol, catepsin B triggers a cascade resulting in the dissociation of NF-kB from its complex with the protein IKB a fact that allows NF-kB to enter into the nucleus and mediate production of proinflammatory cytokines like the TNF-a.

tein, a cytosolic protein that retains the transcription factor NF-κB in the cytoplasm. Following phosphorilation IKB protein dissociates from NF-κB allowing the latter to enter the nucleus. In the nucleus NF-kB mediates induction of proinflammatory cytokines implicated in insulin resistance.[71] Several lines of evidence support the role of TNF/NF-kB-mediated activation of IKKβ as a mechanism for insulin resistance. Mice lacking TNF or TNF receptors (TNF-R) have improved insulin sensitivity in both dietary and genetic models of obesity.[72] High dose salicylate inhibits IKKβ activity[73] and reverses insulin resistance, hyperglycaemia and hyperinsulinaemia in obese and diabetic rodents,[74] while heterozygous depletion of IKKβ protects against the development of insulin resistance during high fat feeding.[75] Conversely, mice with chronic hepatocellular activation of NF-kB, resulting in continuous activation of IKKβ, have insulin resistance and a diabetic phenotype.[76-78]

TNF also affects the insulin signaling pathway in other ways. Firstly the presence of TNF favours IL-6 and related suppressor of cytokine signaling-3 (SOCS-3) production[79] which blocks the activity of the insulin receptor and secondly it inhibits the intracellular insulin signaling pathway by activation of serine phosphorylation of IRS.[10,59] Finally high TNF levels also suppress production and activity of adiponectin [77;80] indicating that both high TNF (more insult) and low adiponectin (less protection) levels, act together in the development of insulin resistance.

Interleukin-6 is the second important proinflammatory cytokine that plays a role in the metabolic syndrome. In non-inflammatory conditions, one third of circulating IL-6 originates from adipose tissue. Circulating IL-6 is strongly associated with obesity and is a predictor of development of type II diabetes. IL-6 is an inhibitor of insulin signaling in isolated hepatocytes and in the liver of experimental animals.[81] In models of genetic or diet-induced obesity, injections of IL-6 neutralizing antibodies allow normalisation of insulin receptor phosphorylation in response to insulin, and increase insulin-mediated suppression of hepatic glucose output.[82] This improvement of insulin signaling is restricted to the liver as glucose uptake and insulin resistance in muscle and adipose tissue are not affected. Whether IL-6 dependent transcriptional activation of SOCS-3 is responsible for the inhibition of the insulin receptor, remains to be proven.

In conclusion during obesity the increasing concentrations of circulating cytokines affects both lipid and insulin signalling pathways which influencing both glucose and lipid metabolism. Additionally it seems that these cytokines also favour recruitment of effector cells like lymphocytes and neutrophils which are frequently found in the liver tissue of patients with steatohepatitis.[83]

CONCLUSIONS

Increase in body weight and intrabdominal fat is associated with alterations in insulin signalling and insulin resistance, lipogenesis and consequently hepatic steatosis; The liver is an insulin sensitive organ that plays a key role in the regulation of the whole body energy homeostasis and hepatic insulin resistance is the main cause of fasting hyperglycemia and hyperinsulinaemia during obesity.

The adipose tissue has been considered as a multi-located organ and is a pathogenic site of obesity-induced insulin resistance, because of metabolic alterations, changes in adipocytokine production and presence of the inflammatory cytokines. Hepatic inflammation and fibrosis may result from the exposure of the fatty liver to metabolic and pro-inflammatory mediators, produced by visceral fat and drained by the portal circulation. In the study by Feldstein et al[68] in human NAFLD livers and an in vivo murine model of fatty liver the presence of increased levels of FFA in the circulation caused lysosomal permeabilization and release of catepsin B in the cytosol of the liver cells causing alterations in the NF-kB pathway and an increase in the production of TNF-a. Especially in patients with moderate to severe steatohepatitis cytosolic localization of catepsin B was significantly increased compared with those with only mild inflammatory activity. Furthermore in the murine model treatment with a selective catepsin B inhibitor improved the histologic appearance of steatohepatitis suggesting that as a possible target for future treatments. In the study by Calvert et al[84] a proteomic analysis identified differences in the livers of patients with NASH versus those with simple steatosis regarding several phosphorylation events which affect mainly the PKC lipolysis pathway and the AKT/mTOR pathway, all part of the insulin signaling pathway. Moreover Ma et al[85] found that in hepatic cells and in the livers of apolipoprotein E knockout mice the presence of high cytokine levels (IL-1b and TNF-a) caused an increase in the intracellular cholesterol levels by up-regulating LDL-receptor mediated cholesterol influx and down-regulating adenosine triphosphate-binding cassette transporter A1(ABCA1) mediated cholesterol efflux showing that disruption in cholesterol trafficking control may play a role in the progression of NAFLD especially during the second hit phase of liver damage.

Understanding the pathogenesis of NAFLD is useful to identify predictors of disease progression, possible genetic predisposing factors, and to clarify the effects and limitations of therapeutic regimens that are already tried in practice. New findings regarding the involvement of specific signaling pathways provide important potential targets for future therapeutic interventions. However more studies are needed before we reach the point to see a reduction in morbidity and mortality from this disease.

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