Insulin resistance and oxidative stress interdependency in non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is emerging as a major cause of chronic liver disease in association with the rising prevalence of obesity and type 2 diabetes in the population. Oxidative stress and insulin resistance (IR) are major contributors in the pathogenesis of NAFLD and in the progression from steatosis to steatohepatitis. Recently, Houstis and colleagues reported that reactive oxygen species have a causal role in multiple forms of IR, a phenomenon that can further promote exacerbation of oxidative stress. The improvement of the knowledge of these interrelationships should contribute to elucidate pathogenic pathways and design effective treatments for NAFLD.

Pathogenic factors in non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is emerging as the most important cause of chronic liver disease in relation to the increasing incidence of obesity and type 2 diabetes in the population. NAFLD encompasses a wide disease spectrum ranging from uncomplicated triacylglycerol (TAG) accumulation in hepatocytes (hepatic steatosis) to steatosis with inflammation, fibrosis and cirrhosis (non-alcoholic steatohepatitis, NASH). The primary metabolic abnormalities that lead to hepatic steatosis and involve a lipotoxic response with an oxidative-stress component include nutritional factors and alterations in the lipid metabolism of the liver, which result from either gene polymorphisms or, more importantly, from the development of insulin resistance (IR) [1]. In this regard, visceral fat has been related to IR and oxidative stress to a greater degree than subcutaneous fat [1]. In the chronic setting, IR might, in turn, exacerbate the oxidative-stress status of the liver, thereby promoting the hepatic necroinflammation and the progression of the disease. Recently, Houstis *et al.* [2] combined gene-expression analysis and measures of redox state in cultured 3T3-L1 pre-adipocytes to show that reactive oxygen species (ROS) have a causal role in multiple forms of IR. Here, we discuss the implications of these findings in relation to oxidative stress–IR interdependency in NAFLD as a crucial factor for elucidating pathogenic mechanisms and designing effective therapies.

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Involvement of oxidative stress in hepatic steatosis and its exacerbation in steatohepatitis

Hepatic fat accumulation in NAFLD associated with obesity might be determined by an increased peripheral lipolytic activity that is secondary to IR, with augmentation of fatty acid (FA) and glycerol fluxes to the liver, and depletion of hepatic long-chain polyunsaturated FAs (LCPUFAs), particularly of the *n-3* series [3]. The depletion of *n-3* LCPUFA might direct FAs away from oxidation and secretion and towards TAG storage because *n-3* LCPUFAs suppress lipogenic gene expression and induce expression of genes related to hepatic lipid oxidation and export from the liver (Box 1).

Non-adipose tissues have limited capacity for TAG storage and, under conditions of overnutrition, excess lipids accumulate determining high levels of saturated FAs that can trigger cell dysfunction and/or cell death, a response known as lipotoxicity [4]. The phenomenon involves an elevation of ROS levels that are produced at complex I of the mitochondrial electron-transport chain [4] and might explain the moderate increase in the oxidative-stress status of the liver in obese patients with steatosis (Figure 1). This redox imbalance is characterized by: (i) a lower than normal antioxidant potential, as evidenced by significant hepatic glutathione (GSH) depletion and reduced superoxide dismutase (SOD) activity; and (ii) an enhanced free-radical activity, as evidenced by the increments in hepatic lipid peroxidation, protein oxidation and 3-nitrotyrosine reactivity [5,6]. Consequently, a 21% decrease in the systemic antioxidant capacity of plasma is elicited [3]. The redox disequilibrium described above represents a nutritional oxidative-stress phenomenon, resulting from prolonged excess oxidative load (carbohydrates and lipids) and/or inadequate nutrient supply (dietary antioxidants) favoring pro-oxidant reactions [7].

Diminished liver SOD activity and GSH levels, and increased lipid peroxidation in obese patients with steatosis persist in those with steatohepatitis. In steatohepatitis these features are accompanied by: (i) low catalase activity; (ii) high 8-hydroxydeoxyguanosine content as a marker of oxidative DNA damage; (iii) a further increment in 3-nitrotyrosine reactivity; and (iv) induction of inducible nitric-oxide synthase and cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1) [5,6]. These hepatic changes are paralleled by significant increases in malondialdehyde and 4-hydroxynonenal levels in the serum, and

Box 1. Liver long-chain polyunsaturated fatty acid (LCPUFA) signaling in NAFLD

- Livers of obese NAFLD patients with triacylglycerol (TAG) accumulation exhibit lower levels of LCPUFA, particularly of the *n-3* series [eicosapentaenoic acid (20:5, *n-3*) and docosahexaenoic acid (22:6, *n-3*)], compared with those of controls.
- Liver *n-3* LCPUFA depletion might be caused by several mechanisms including: (i) lower-than-normal dietary consumption of the essential precursor α-linolenic acid (18:3, *n-3*); (ii) defective desaturation and elongation of PUFA, as evidenced by the lower liver product/precursor ratio [(20:5 + 22:6) *n-3*/18:3, *n-3*]; (iii) higher-than-normal dietary consumption of *trans*-fatty acids, such as elaidic acid (18:1, *n-9 trans*), which exert a substantial inhibitory action on hepatic Δ-6 desaturase activity; and/or (iv) increased peroxidation of LCPUFA due to oxidative stress (Figure 1).
- *n-3* LCPUFAs are signaling biomolecules regulating lipid metabolism through: (i) downregulation of the expression of SREBP-1 and its processing, with inhibition of the transcription of lipogenic and glycolytic genes; and (ii) upregulation of the expression of genes that encode enzymes of fatty acid oxidation, acting as ligand activators of PPAR-α. Therefore, depletion of *n-3* LCPUFA in the liver of NAFLD patients might favor fatty acid and TAG synthesis over fatty acid oxidation, leading to fatty liver.
- Reduction in TAG export from the liver is also likely to occur under conditions of *n-3* LCPUFA depletion, considering that PPAR-α activation by LCPUFA increases the production of apolipoprotein B-100, a rate limiting step in hepatic very low density lipoprotein (VLDL) formation, and induces the transcription of liver fatty acid-binding protein, an acceptor of long-chain FAs that enhances their uptake and intracellular transport, and the assembly and secretion of VLDL.

a further reduction in the antioxidant capacity of the plasma in steatohepatitis over steatosis [5,6]. Collectively, these findings point to an increase in the oxidative-stress status of the liver in steatohepatitis compared with that of steatosis, which is coincident with the upregulation of CYP2E1 expression (Figure 1). From a pathological point of view, CYP2E1 induction in NASH is considered of particular relevance because of its substantial pro-oxidant activity [8], a response that is caused by significant attenuation of the repressive effect of insulin on CYP2E1 expression exerted either at transcriptional or post-transcriptional level [9]. CYP2E1 expression might be favored by the presence of specific relevant polymorphic sites in the *CYP2E1* gene, promoting elevated transcriptional activity, protein expression and enzyme activity, such as the rare mutant c2 allele that lacks the *Rsa1* restriction site [8] (Figure 1). Therefore, induction of liver microsomal CYP2E1, which is related to liver injury in NADLF patients [10], constitutes a major free-radical source that is associated with increased oxidative stress prevailing in NASH, although other mechanisms cannot be ruled out. These include elevated ROS production associated with: (i) mitochondrial dysfunction related to lipotoxicity; (ii) induction of other pro-oxidant isoforms of cytochrome P450 (e.g. CYP4A); and/or (iii) stimulation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity of Kupffer cells and/or polymorphonuclear and mononuclear cells that infiltrate the liver (Figure 1). Exacerbation of oxidative stress in NASH might promote hepatocellular damage and death by inducing severe oxidative alterations in biomolecules with loss of their functions and impairment of cell viability. Alternatively, activation of redox-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) might also occur, with consequent upregulation of the expression of pro-inflammatory mediators (Figure 1).

Oxidative stress and IR interdependency

The interdependency between oxidative stress and IR might arise from a pathophysiological sequence of reinforcing mechanisms. The data reported by Houstis *et al.* [2] established that increases in ROS levels precede the onset of IR and might be causally linked to it. Although these findings were reported in cultured adipocytes, it is expected that they also occur in hepatocytes because the primary metabolic abnormalities in these cells also induce ROS production, thus triggering the onset of IR [2,4]. This is evidenced by a 2.2-fold increase in the homeostasis model assessment (HOMA) index of IR in obese patients with steatosis over control values [3]. Indeed, persistent ROS generation and/or elevated levels of free saturated FAs can diminish insulin action through activation of serine–threonine kinase cascades that, in turn, phosphorylate several targets, including the insulin receptor and the insulin receptor substrate (IRS) proteins, with consequent decrease in insulin-stimulated tyrosine phosphorylation [11]. In addition, IR in NAFLD might be related to: (i) enhancement in the intrahepatic and intramyocellular lipid content [12]; and/or (ii) depletion of *n-3* LCPUFAs [3] because these are expected to modify membrane-mediated processes such as insulin signaling [13] (Figure 1).

The progression of steatosis to steatohepatitis is associated with exacerbation of liver oxidative stress, mainly due to IR-dependent CYP2E1 upregulation, a response that is also facilitated by excess saturated FAs and ketones [8], and a concomitant enhancement in the

Box 2. Pro-inflammatory cytokine signaling in NASH

- NASH patients exhibit higher-than-normal serum levels of tumor necrosis factor-α (TNF-α) associated with increased prevalence of small intestinal overgrowth compared with that of controls, without changes in intestinal permeability or endotoxemia.
- NASH patients with increased serum TNF-α concentrations also show higher levels of interleukin (IL)-6 and chemokine IL-8.
- Severely obese patients with NASH overexpress TNF-α mRNA both in liver and adipose tissue, and higher hepatic levels of p55 TNF-α receptor mRNA. These changes are more dramatic in patients with more advanced NASH.
- In addition, increase of TNF-α and soluble TNF-α receptor 2 levels in the serum of NASH patients is associated with decreased levels of adiponectin and reduced hepatic expression of adiponectin and adiponectin receptor-II, an adipokine having significant anti-inflammatory and metabolic effects that prevent liver disease.
- Interestingly, increased TNF-α and IL-1α production by lipopolysaccharide (LPS)-stimulated whole-blood monocytes and/or macrophages occurs in NAFLD patients, a response that is more marked in steatohepatitis than in steatosis and that correlates with the respective histology scores. Overproduction of IL-6 and IL-8 has also been observed in LPS-stimulated isolated monocytes. These findings might support a pathophysiological role for the above-mentioned cytokines in the production of NASH upon inflammatory leukocyte infiltration of the liver.

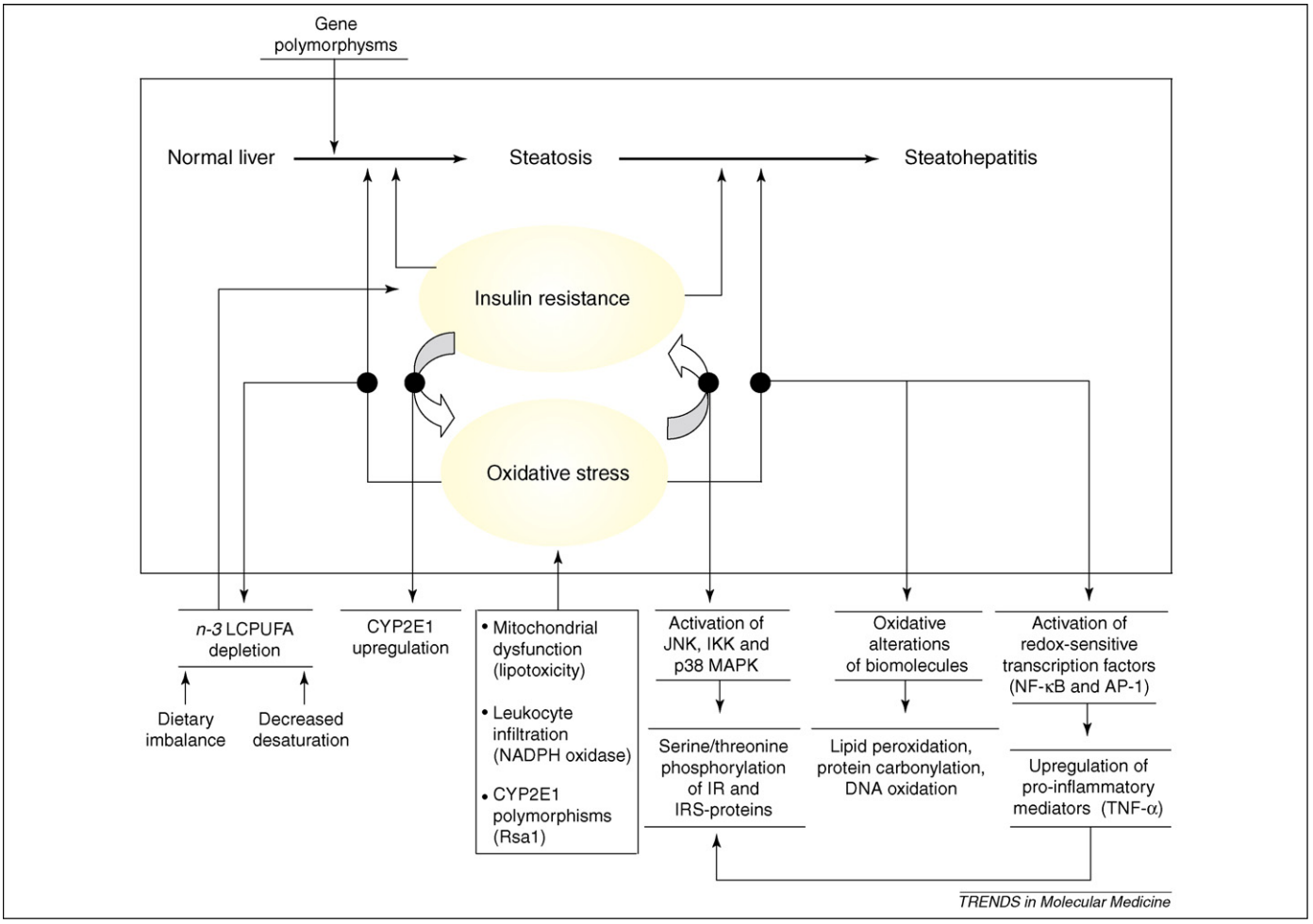

Figure 1. Interrelationships between oxidative stress and IR mechanisms in NAFLD associated with obesity. The initial FA overloading of the liver can increase mitochondrial ROS production (lipotoxicity) leading to: (i) depletion of *n-3* LCPUFA, which directs FAs away from oxidation and secretion and towards TAG storage, promoting hepatic steatosis; and (ii) the onset of IR, as shown by Houstis *et al.* [2] through ROS- and FA-induced activation of serine–threonine kinases that phosphorylate the insulin receptor and insulin receptor substrate proteins, thus decreasing insulin signaling. The progression of steatosis to steatohepatitis is associated with: (i) exacerbation of oxidative-stress status due to IR-dependent CYP2E1 upregulation, mitochondrial dysfunction and Kupffer cell or infiltrating leukocyte NADPH oxidase activity; and (ii) enhancement of IR due to the reinforcement of serine–threonine phosphorylation of components of the insulin cascade, which might be triggered by the upregulation of pro-inflammatory mediators (TNF-α) through activation of the redox-sensitive transcription factors (NF-κB and AP-1) under conditions of excessive and prolonged oxidative stress. Abbreviations: AP-1, activator protein-1; CYP2E1, cytochrome P450 isoform 2E1; IKK, inhibitor of κB kinase; IR, insulin receptor; IRS, insulin receptor substrate protein; JNK, c-Jun N-terminal kinase; LCPUFA, long-chain polyunsaturated fatty acid; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α. .

HOMA index (3.4-fold higher than control values; 53% higher than steatosis values) [3]. Under conditions of significant and prolonged oxidative stress, upregulation of pro-inflammatory mediators can occur [1,14] (Box 2) through sustained redox activation of NF-κB or AP-1 in Kupffer cells [15]. In a chronic inflammatory state, enhanced expression and release of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1α (Box 2) might stimulate the activity of IRS-1-associated c-Jun N-terminal kinase (JNK) and other serine–threonine stress kinases [stress-activated protein kinase (SAPK), inhibitor of κB kinase (IKK) and protein kinase C-θ (PKC-θ)], resulting in increased serine phosphorylation of the insulin receptor and IRS-1 [11,16]. Enhancement in serine phosphorylation can lead to impaired insulin action by reducing tyrosine phosphorylation of the insulin receptor and IRS-1, and might increase proteasome-mediated IRS-1 degradation, thus further suppressing downstream signaling [11]. Collectively, the discussed evidence supports the view of a functional interdependence between oxidative stress and IR (Figure 1). This might involve initial ROS production and lipotoxicity, in association with the onset of IR in steatosis, and a further enhancement in ROS generation due to CYP2E1 induction, lipotoxicity, Kupffer cell or infiltrating phagocyte NADPH oxidase activity, and/or mitochondrial dysfunction that is present in steatohepatitis [1,5]. In addition, the redox upregulation of TNF-α and IL-1α that reinforces the initial mechanisms of IR and ROS production might also have a role. Under these conditions, antioxidants that suppress oxidative stress by lowering ROS levels can act as insulin sensitizers, in addition to

Box 3. Outstanding questions

- Is the onset of IR triggered by ROS *in vivo*?
- Do antioxidants ameliorate IR and/or oxidative stress in NAFLD?
- Do the metabolic transcription factors PPAR-α and SREBP-1 account for the alterations of lipid metabolism leading to hepatic steatosis in NAFLD?
- Is the expression and/or activation of the hepatic pro-inflammatory transcription factors NF-κB and AP-1 altered in NASH?
- What are the signaling-cascade sequences determining the progression from steatosis to steatohepatitis?

abrogating free-radical-mediated damage to biomolecules, as shown by Houstis *et al.* [2] in cell-culture studies. Nevertheless, these findings remain to be confirmed *in vivo* (Box 3).

Concluding remarks

NAFLD in obese patients underlie oxidative stress and IR, which seem to be interdependent, a proposal that deserves further investigation on the mechanistic aspects of the signaling cascades that are involved in the pathogenic pathways of liver damage. These include the expression and/or activation status of pro-inflammatory (NF-κB and AP-1) and metabolic [peroxisome proliferator-activated receptor-α (PPAR-α) and sterol regulatory element binding protein-1 (SREBP-1)] transcription factors, and the activity of serine–threonine stress kinases, all contributing to understand the role of chronic and progressive oxidative stress and inflammation in determining IR in human NAFLD (Box 3). The importance of the above contention is highlighted by the lack of a drug therapy that is effective for NAFLD at present [1,8,11], suggesting potentially attractive therapeutic targets to improve insulin sensitivity in obese NAFLD patients. In this respect, it has been recently reported that weight loss improves the key features of NAFLD and NASH inducting: (i) a significant reduction of the steatosis score and IR index [17,18]; (ii) normalization of associated metabolic abnormalities; and (iii) reduction in the hepatic expression of pro-inflammatory and fibrogenic mediators [18]. IR is a major issue in the metabolic syndrome that is causally associated with the liver condition of obese patients, thus clearly constituting a hepatotoxic condition. These considerations strongly suggest that weight reduction, as a central therapeutic issue, might be combined with antioxidants, agents that suppress the chronic changes in the signaling cascades involved (*n-3* LCPUFA) [5] and/or innocuous CYP2E1 inhibitors (dilinoleoylphosphatidylcholine) [19] to reverse or prevent the onset of oxidative-stress-induced inflammatory response and IR in NAFLD. Thus, determination of both membrane LCPUFA composition and reliable indicators of oxidative stress ought to be assessed in future studies, in addition to the clinical and biochemical variables usually analyzed. These might include: (i) the ferric reducing ability of the plasma as index of antioxidant capacity; (ii) the *in vivo* hydroxylation of chlorzoxazone, which measures the activity of the pro-oxidant enzyme CYP2E1; and/or (iii) the serum levels of malondialdehyde, 4-hydroxynonenal, or F_2-isoprostanes, which are evidence of free radical-mediated polyunsaturated fatty acids (PUFA) peroxidation. The above assays that assess oxidative-stress status have been recently validated in NAFLD patients [3,6,10].

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