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FORUM REVIEW ARTICLE

Adipokines and Redox Signaling: Impact on Fatty Liver Disease

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Abstract

Adipokines (adipose tissue cytokines) are polypeptide factors secreted by adipose tissue in a highly regulated manner. The 'classical' adipokines (leptin, adiponectin, and resistin) are expressed only by adipocytes, but other adipokines have been shown to be released by resident and infiltrating macrophages, as well as by components of the vascular stroma. Indeed, adipose tissue inflammation is known to be associated with a modification in the pattern of adipokine secretion. Several studies indicate that adipokines can interfere with hepatic injury associated with fatty infiltration, differentially modulating steatosis, inflammation, and fibrosis. Moreover, plasma levels of adipokines have been investigated in patients with nonalcoholic fatty liver disease in order to establish correlations with the underlying state of insulin resistance and with the type and severity of hepatic damage. In this Forum article, we provide a review of recent data that suggest a significant role for oxidative stress, reactive oxygen species, and redox signaling in mediating actions of adipokines that are relevant in the pathogenesis of nonalcoholic fatty liver disease, including hepatic insulin resistance, inflammation, and fibrosis. *Antioxid. Redox Signal.* 15, 461–483.

Adipokines

White adipose tissue, the most abundant in adults, has three main functions: 1) storage of energy in the form of triglycerides; 2) hydrolysis of triglycerides to provide free fatty acids to support the energy needs of tissues, especially skeletal muscle; 3) release of adipokines (or adipocytokines). Data accumulated over the last two decades have shown that the functions of adipose tissue are not limited to energy storage, but extend to fundamental processes such as metabolism, immune response, tissue regeneration, wound healing, and cancer. In this context, the relations between the liver and adipose tissue are particularly close, and represent the result of a diversification of functions that are present in the same tissue in lower organisms (72). The interest in adipose tissue pathophysiology has grown along with the dramatic increase in the prevalence of obesity (116). Obesity is a component of the metabolic syndrome and thus a major risk factor for the development of nonalcoholic fatty liver disease (NAFLD) and for progression to advanced fibrosis and cirrhosis in the context of nonalcoholic steatohepatitis. Nonetheless, adipose tissue expansion has an impact on liver diseases in general, as cirrhosis is more prevalent in obese patients (139), and obesity favors the appearance of severe fibrosis in chronic liver diseases (3), and confers a higher risk of liver cancer (28).

Adipokines (adipose tissue cytokines) are polypeptide factors which are expressed significantly, although not exclusively, by adipose tissue in a regulated manner. In adipose tissue, adipokines modulate adipocyte differentiation and regulate lipid accumulation through autocrine mechanisms. This feature has a clear relevance in obesity that is associated with adipose tissue inflammation, and has been linked to the appearance of insulin resistance and to the metabolic and cardiovascular complications of the metabolic syndrome (153). An additional factor that has received considerable attention in the pathophysiology of adipose tissue is the role of ectopic fat, that is, adipose tissue expansion at sites different from subcutaneous adipose tissue, such as in the omentum (visceral fat) or around the heart (epicardial or mediastinal fat). Ectopic fat is more likely to undergo inflammation and to contribute to the pathogenesis of obesity-related disorders (72).

Only one-third of adipose tissue is composed of adipocytes, the rest being stromal cells, macrophages, fibroblasts, and monocytes, all of which contribute to adipokine production. The 'classical' adipokines are those primarily expressed by adipocytes, namely leptin, adiponectin, and resistin, but an expanding group of newly identified adipokines is currently

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being investigated and some of them may be expressed by vasculo-stromal cells (117).

Leptin has been the first adipokine to be identified, as the circulating product of the *obese (ob)* gene, which is expressed by adipose tissue and at several other sites (129). Leptin receptors belong to the class I of cytokine receptor superfamily, and six isoforms have been identified. The long receptor, ObRb, mediates most of the biological effects of this adipokine, and is able to fully activate intracellular signaling via the Jak-2/Stat3 pathway (129). Secretion of leptin is directly proportional to the fat mass, and provides anti-obesity signals, regulating food intake and energy expenditure in conditions of energy excess, through hypothalamic pathways. This action is reflected by the phenotype of *ob/ob* mice, which lack functional leptin and are obese and hyperphagic. In addition, leptin stimulates wound repair, modulates innate and adaptive immunity, and regulates hematopoiesis and reproduction.

Adiponectin circulates as a full-length molecule assembled in complexes of different molecular weight, or as the isolated C-terminal globular domain (85). Adiponectin binds at least two specific receptors, AdipoR1 and AdipoR2, (38) which belong to the seven transmembrane domain receptor family but are not coupled to G protein. AdipoR1 is expressed in several tissues and in skeletal muscle, while AdipoR2 is mostly expressed by hepatocytes. Binding experiments indicate that AdipoR1 interacts preferentially with the globular domain, and its main downstream effector is the AMP-activated protein kinase (AMPK). AdipoR2 binds with similar affinity both full-length and globular adiponectin and signals predominantly via activation of peroxisome proliferator-activated receptor-α (PPARα).

Adiponectin concentrations are inversely correlated with fat mass, and are downregulated in patients with obesity (85). Moreover, adiponectin exerts insulin sensitizing effects and acts as an anti-inflammatory molecule. Conversely, inflammation is a potent inhibitor of adiponectin release, and adipose tissue inflammation is considered one of the main mechanisms underlying reduced plasma levels in obesity.

Resistin belongs to a family of small cysteine-rich secretory proteins, named FIZZ (found in inflammatory zone) or RELMs (resistin-like molecules) (166). In rodents, resistin is highly expressed by adipose tissue and circulating levels are increased during diet-induced or genetic obesity (165), where resistin may be a link between obesity and insulin resistance (137). Reduced hepatic glucose production in the absence of resistin is also associated with higher hepatic AMPK activation (14). In humans, the biology of resistin is less defined, and most studies demonstrate that resistin is expressed predominantly by bone marrow-derived cells and inflammatory cells (128). In human monocytes, resistin expression is increased by treatment with proinflammatory cytokines *in vitro* and circulating levels of resistin are higher in different conditions of inflammation.

Adipokines, Oxidative Stress, and Hepatic Insulin Resistance

Insulin signaling pathways and the hepatic control of glucose and lipid metabolism

Hepatocytes, together with myocytes and adipocytes, represent a major cellular target for insulin by expressing a high number of insulin receptor (IR) molecules on their plasma membrane (32, 175). The most relevant aspects of the insulin signaling network will be briefly summarized herein and in Figures 1 and 2, dedicated to the overall control of insulin signaling (110, 123, 175, 187) and related cellular responses (175), respectively. Insulin, binding to one of the two isoforms of its tetrameric receptor (IR_A or IR_B, having different affinity for insulin and IGF1), activates signaling by leading to de-repression of tyrosine-kinase (TK) activity of the two intracellular β-subunits and to trans-phosphorylation of specific tyrosine residues, resulting in a further increase in kinase activity.

Following activation of IR (and IGFR1), the insulin signaling network, which is carefully controlled by a number of mechanisms (illustrated in Fig. 1, Ref.175), proceeds through phosphorylation of intracellular substrate proteins, including the six different isoforms of insulin receptor substrate (IRS) proteins (IRS 1–6), and various isoforms of Src homology-2 (SH2)-containing proteins (175), that in turn, mediate the binding of downstream effectors. Best characterized and most relevant for insulin signaling are IRS1 and IRS2 (widely distributed, major isoforms in hepatocytes), IRS3 (mostly restricted to adipose tissue and brain), and IRS4 (expressed in embryos and cell lines). Phosphorylated IRS serve as docking platforms for signaling proteins containing SH2, including: 1) the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) or the adaptor Grb2, which activate the Ras/MAPK signaling pathway; 2) enzymes like SH2-domain containing tyrosine phosphatase-2 (SHP2), cytoplasmic tyrosine kinases, and the Ca2+-ATPases SERCA1 and 2.

IR activation, through the involvement of IRS, results in the engagement of two main divergent pathways (Fig. 2; 175): 1) PI3K, leading to activation of PKB/Akt or atypical PKC isoforms, a pathway which mediates the main metabolic actions of insulin with a complexity that is exacerbated by the existence of multiple isoforms of IRS, PI3K, (regulatory and catalytic) and Akt (reviewed in Ref. 175); 2) the MAP kinase pathway, leading to cell proliferation, survival, and differentiation.

In hepatocytes (67, 99, 175), IRS-1 has a major role in handling increased glucose levels in the fed state and is involved in the control of glycogen synthesis and lipogenesis. Glucose, entering hepatocytes through the specific GLUT-2 transporter, is then directed to the glycolytic pathway and ATP production in order to fulfill energy requirements and replenish glycogen stores. Excess glucose can be diverted to lipogenesis, and insulin can then act as a potent stimulator of lipogenic pathways by operating mainly through sterol regulatory element binding protein-1 (SREBP-1) and the glucose-responsive transcription factor carbohydrate response element-binding protein (ChREBP) (135, 178). IRS2 is more involved in the fasting state, when it is upregulated to favor the action of insulin, limiting the expression of gluconeogenic enzymes such as glucose-6-phosphatase or phosphoenolpyruvate carboxykinase (PEPCK) and then the hepatic production of glucose and its export by the GLUT2 transporter. Besides the opposing effects of insulin and glucagon on fatty acid mitochondrial oxidation, insulin signaling limits glucagon-stimulated gluconeogenesis by means of Akt–dependent phosphorylation of the forkhead box–containing protein O subfamily-1 (FOXO1) transcription factor. When phosphorylated, FOXO1 is segregated into the cytoplasm and degraded by the proteasome pathway, preventing nuclear translocation and regulation of PEPCK expression (99, 135).

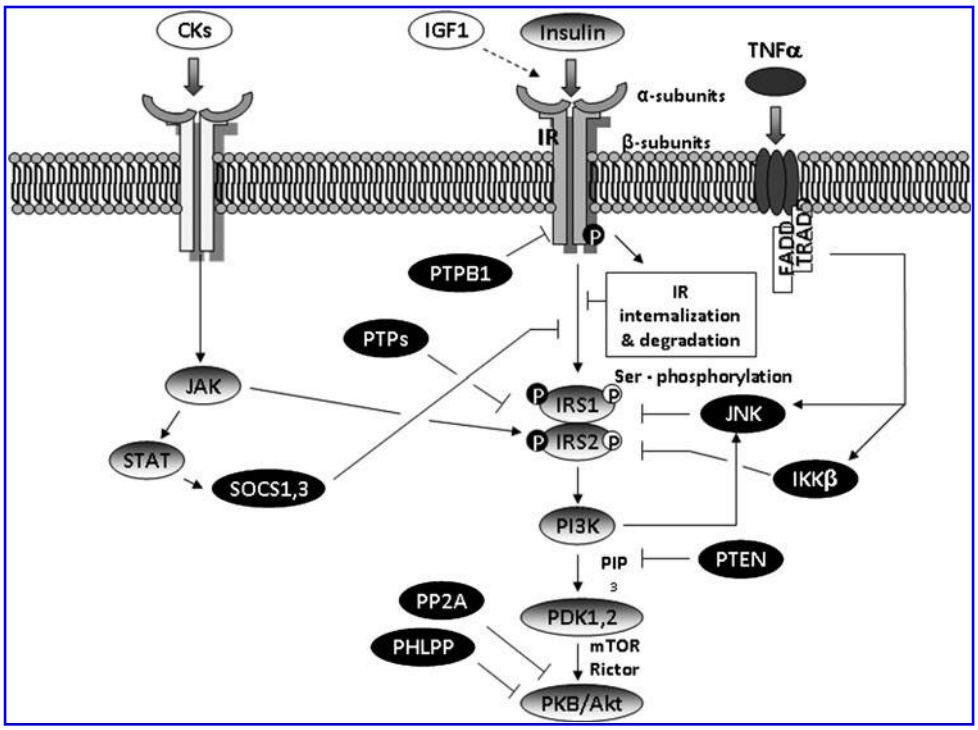

FIG. 1. Insulin signaling pathway and its regulatory mechanisms. The figure shows the major steps in insulin-mediated signaling following interaction of insulin with the tetrameric insulin receptor (IR) complex at the plasma membrane level, together with the major inhibitory mechanisms and the cross-talk with signaling pathways elicited by cytokines and growth factors, including TNFα and IGF-1. Following ligand–receptor interaction, IR activation is tightly regulated by several negative mechanisms that include (175): a) tyrosine phosphatases, particularly PTP1B that directly interacts with IR to dephosphorylate critical tyrosine residues; b) proteins that sterically block IR function either by preventing its interaction with intracellular substrate proteins or by modifying its kinase activity, including suppressor of cytokine signaling (SOCS)-1 and −3, which are known to be upregulated under conditions of insulin resistance; and c) ligand-stimulated internalization and degradation of IR, a process which again occurs under conditions of insulin resistance.

Upon activation, IR as well as IGF1R can phosphorylate intracellular substrate proteins that mediate the binding of downstream effectors, the most relevant being the isoforms of insulin receptor substrate (IRS) proteins, leading then to the activation of PI3K and PKB/Akt. PI3K acts as a negative regulator of insulin signaling on the basis of at least three distinct mechanisms: 1) efficiency of insulin signaling critically depends on the stoichiometry of the p85 regulatory subunit to the catalytic heterodimer (123); 2) sequestration of IRS/PI3K complex into signaling-silent compartments (*i.e.*, incapable of generating PIP3) provides negative regulation (110); 3) the recruitment of inhibitory proteins that interact with monomeric p85α regulatory subunits. This leads to either PIP3 degradation by phospholipid phosphatases like PTEN or through negative regulation of insulin signaling by activation of JNK activity. In particular, JNK activation, which is also induced by TNF, operates its inhibitory action phosphorylating IRS1 and IRS2 on serine residues (175, 187). A very close mechanism has been described for IκB kinase beta (IKKβ) that controls the activation of NF-κB; once activated, IKKβ phosphorylates IRS-1 on Ser307 residue, resulting in inhibition of insulin action (63). Further mechanisms leading to inhibition of insulin signaling are operated through suppressors of cytokine signaling (SOCSs), regulated by several cytokines, including IL-6 and leptin, and upregulated in conditions of insulin resistance (135, 175, 178). SOCS proteins act by linking IRS to ubiquitin-mediated degradation pathways and activating SREBP1, thus favoring lipogenesis (154, 188). Similar SOCS-mediated mechanisms may operate in insulin resistance associated with chronic HCV infection, as HCV core protein can upregulate SOCS and then promote degradation of IRS1 and IRS2 (91).

Akt is negatively regulated by the action of the phosphatases Src-homology-2 domain-containing inositol phospatase-2A (PP2A) and the pleckstrin-homology (PH) domain leucine-rich-repeat protein phosphatase (PHLPP). Other details can be found in the text.

As far as PI3K and Akt are concerned, IR- or IGF1R-related PI3K involvement results from direct interaction with phosphorylated substrate proteins, including IRS, leading to phosphatydylinositol-3-phosphate (PIP_3) generation and, in turn, PIP_3-mediated activation of different Akt isoforms. Apart from negative feedback control on insulin signaling by PI3K (see Fig. 1), PIP3 recruits phosphoinositide-dependent kinase-1 and Akt isoforms at the plasma membrane, where the different Akt isoforms (Akt-1, −2, and −3) are phosphorylated on tyrosine and serine residues by PDK1 and PDK2, possibly through formation of a complex with the mammalian target of rapamycin (mTOR). Phosphorylation of Akt in turn confers the ability to phosphorylate a series of downstream signaling targets that mediate major effects of insulin signaling on glycogen synthesis, gluconeogenesis, and protein synthesis (175).

Hepatic insulin resistance and its effects on glucose and lipid metabolism

It has been proposed that hepatic insulin resistance represents the single major pathophysiologic derangement in the metabolic syndrome (95) as well as a major culprit in the development of type II diabetes (59, 184). Along these lines, nonalcoholic fatty liver disease (NAFLD) is strongly associated with both hepatic and adipose tissue insulin resistance, and reduced whole body insulin sensitivity (26, 115, 156). Moreover, NAFLD patients also exhibit a reduction in fatty acid oxidation, a feature which is likely to reflect a decreased uptake and utilization of glucose as a source of energy (26). All these findings have led to the suggestion (see Ref. 135) that insulin resistance may represent an intrinsic defect in NAFLD and that the decreased insulin responsiveness at the level of adipocytes may significantly

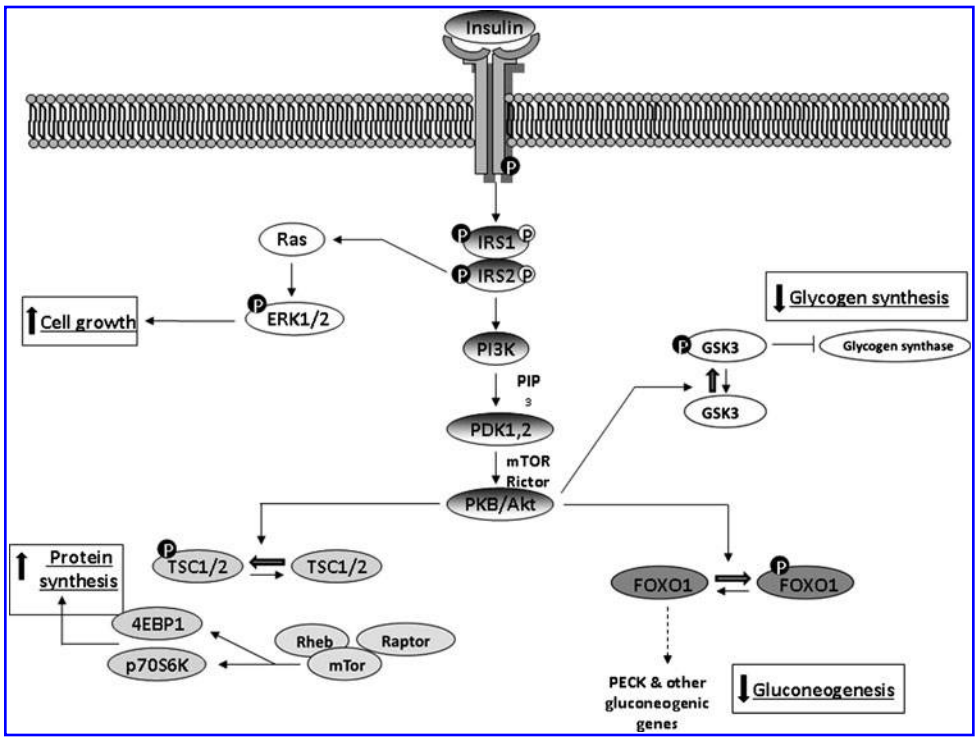

FIG. 2. Insulin signaling and related metabolic or functional responses. IR activation, through the involvement of IRS, results in the engagement of two main and divergent pathways (175). First, the MAP kinase pathway, particularly Ras/Erk, leads to cell proliferation, survival, and differentiation. Second, the PI3K pathway leads to activation of PKB/Akt or atypical PKC isoforms, which mediates the main metabolic actions of insulin. Activation/phosphorylation of PKB/Akt, in particular, confers the ability to phosphorylate a series of downstream signaling targets that mediate major effect of insulin signaling on glycogen synthesis, gluconeogenesis, and protein synthesis: a) glycogen-synthase kinase 3 (GSK3), that when phosphorylated is inactive on glycogen synthase, leading to increased glycogen synthesis; b) FOXO1, a transcription factor which is phosphorylated on Ser256 and sequestered in the cytoplasm, thus preventing its action on genes related to gluconeogenesis like PEPCK; c) Akt substrate of 160 kDa (AS160), which in turn controls the activity of Rab-GTpase activating protein and translocation of glucose transporters (*e.g.*, GLUT4) to plasma membrane; d) tuberin or tuberous sclerosis complex-2 (TSC2) which is complexed with TSC-1: phosphorylation of TSC1/2 by PKB/Akt removes the TSC1/2-mediated inhibitory control on the mTOR pathway, leading to phosphorylation of mTOR downstream signaling substrates like the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (p70S6K), leading to upregulation of protein synthesis.

contribute to hepatic steatosis, providing an excess of FFA flux to the liver. According to the scope of this Forum article, we will focus on the pathophysiology of impaired glucose and lipid metabolism at hepatic level, considering the liver as a major site of insulin action, concentrating our attention on those adipokine- and/or oxidative stress-related mechanisms or molecular derangements associated with hepatic insulin resistance that are also likely to favor the development of liver steatosis.

The major and well-established general problem with insulin resistance is a significant decrease in the ability of insulin to stimulate glucose disposal and inhibit hepatic glucose production (86). It has been suggested (135) that humans can accumulate the excess of FFA as TG stores as a consequence of a limited number of events, including: a) reduction of fatty acid oxidation; b) increased *de novo* lipogenesis; c) increased fatty acid hepatic influx; d) impaired fatty acid and TG efflux from the liver. Moreover, peripheral (*i.e.*, skeletal muscle and adipose tissue) insulin resistance results in an increased flux of FFA towards the liver and then favoring hepatic steatosis (135). Finally, as we will detail later, one should remember that hepatic steatosis itself can further result in insulin resistance and exacerbate whole body insulin resistance (92, 93).

Adipokines, oxidative stress and inflammation: molecular links to hepatic insulin resistance

A derangement or loss of insulin signaling at the hepatocyte level has been proposed to lead to severe insulin resistance and progressive hepatic dysfunction, and is considered a major mechanism for impaired glucose handling and increased fat storage, not only for the liver but also for skeletal muscle and adipose tissue (124). Here we will focus on the role of the adipokines and oxidative stress, and particularly on the role of intracellular levels of reactive oxygen species (ROS), both as mediators and modulators of critical signaling event. An extensive introduction on ROS, antioxidant defenses, and principles of redox signaling is of course out of the scope of the present Forum article and the interested reader can refer to more comprehensive and specialized reviews (37, 132, 177) as well as to Figures 3–6 and related legends which offer, in this connection, a synthetic overview of most relevant concepts. In this review we will try instead to emphasize established and putative redox mechanisms that may be based on ROS or other oxidative stress-related reactive intermediates generated within the cell or entering target cells (mainly hydrogen peroxide and 4-hydroxy-2,3,-nonenal or HNE), that are generated in a pro-oxidant microenvironment.

Intracellular oxidative stress can occur in fat-laden hepatocytes. As reviewed elsewhere (133, 135) increased hepatocyte levels of FFAs in NAFLD/NASH are considered a relevant mechanism leading to hepatocyte injury: a) FFAs can directly induce hepatocyte apoptosis and stimulate production of TNF, which in this context should also be considered as an adipocytokine; b) FFAs can increase Fas ligand binding to the Fas receptor (CD95) in steatotic hepatocytes, leading to apoptosis; c) excess of FFAs in hepatocytes leads to impaired mitochon-

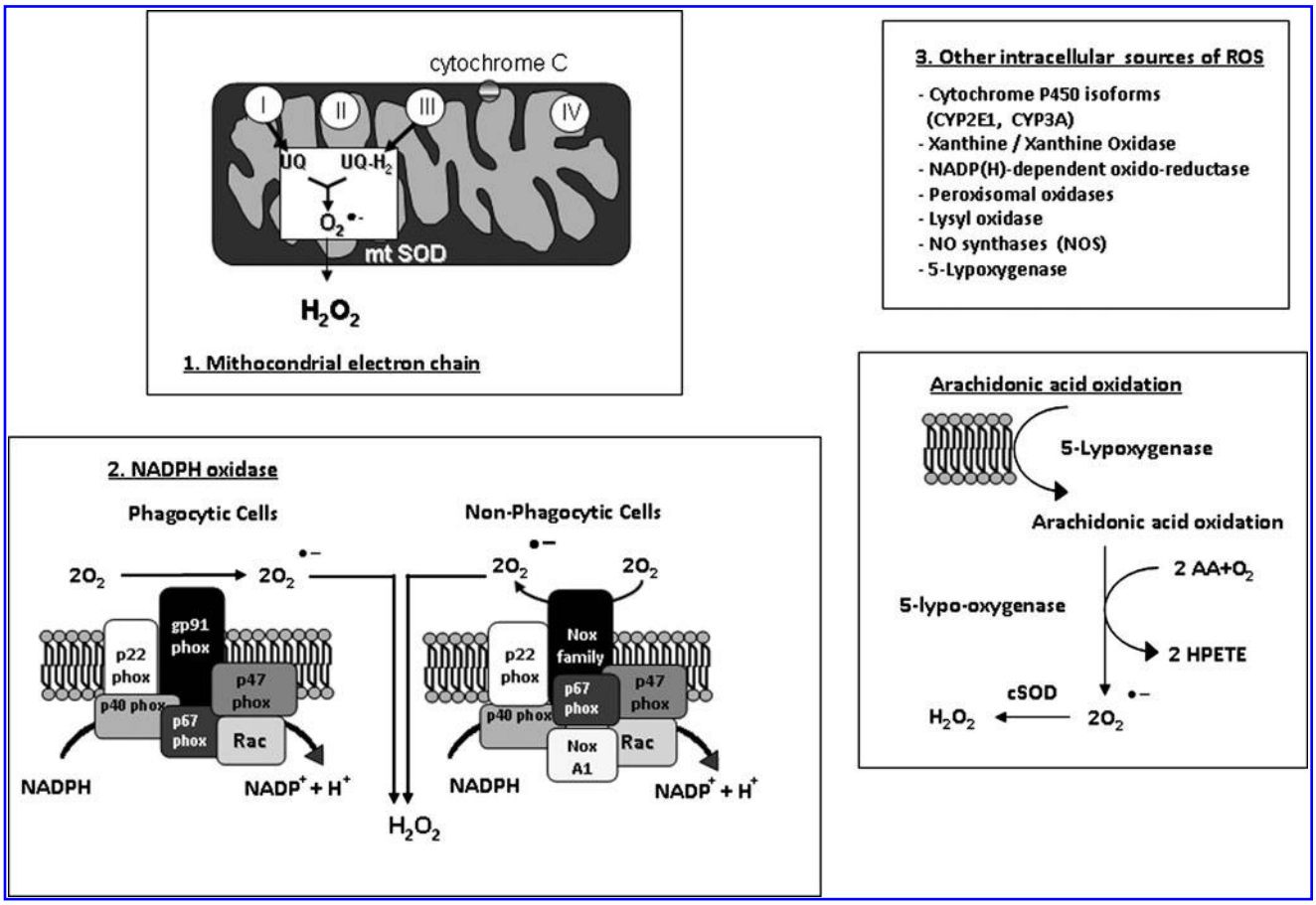

FIG. 3. Intracellular generation of ROS in mammalian cells. ROS can be generated within living cells by the following major sources: 1) Mitochondria: Approximately 1%–5 % of the electrons flowing through the electron transport chain can be diverted to form $O_2^{\bullet -}$ at the level of Complex I (NADH/ubiquinone oxidoreductase) and Complex III (ubiquinol/cytochrome-c oxidoreductase). $O_2^{\bullet -}$ is then converted by a mitochondrial isoform of superoxide dismutase (mtSOD) into H_2O_2 that can cross mitochondrial membranes and then reach the cytoplasm. 2) Plasma membrane NADPH oxidase (NOX): This multi-subunit complex is expressed by professional phagocytic cells (macrophages, neutrophils, and eosinophils) as well as by a number of nonphagocytic cells playing a critical role in human diseases. NOX of professional phagocytes and of nonphagocytic cells are similar in their structure being formed by two membrane bound components (p22phox and gp91phox/Nox2 or another member of the NOX family of protein) forming the flavocytochrome b558, and four cytosolic components (p40 phox, p47phox, p67phox, and the GTPase Rac1/2), that following stimulation, are recruited to the plasma membrane where they interact with Cyt b558 leading to increased activity and then generation of $O_2^{\bullet -}$ that is then converted into H_2O_2. Where redox signaling is concerned, the major difference is that nonphagocytic NOX, which is constitutively active and produce a very low level of ROS, can significantly increase both activity and ROS generation in response to growth factors, cyto- and chemokines, and other conditions. 3) Several enzymes involved in redox reactions: Several enzymes are able to generate ROS (mostly $O_2^{\bullet -}$ that is rapidly converted by a SOD isoform into H_2O_2) during their catalytic activity, including several oxidases, peroxidases, cytochromes, mono- and di-oxygenases, with the following being the most relevant examples: isoforms of the cytochrome P450 superfamily, involved in metabolism of endo- and xenobiotics, including ethanol, steroid hormones, drugs, and chemotherapeutics; xanthine oxidase; the isoforms of cyclooxygenase; peroxisomal oxidases, that can generate directly H_2O_2 when metabolizing various substrates (glycolate-, D-amino-, ureate-, fatty acid-CoA-, and L-α-hydroxyacid oxidases); lysyl oxidase that again generate H_2O_2 when catalyzing the formation of aldehyde precursors of cross-links in collagen and elastin; 5-lipoxygenase, a mixed function oxidase involved in the synthesis of leukotrienes from arachidonic acid in response to stimuli that are also able to stimulate NOX, particularly growth factors and cytokines.

drial or peroxisomal oxidation of FFAs, eventually leading to generation of ROS and products of lipid peroxidation, mainly HNE, which in turn may cause cell injury and death; d) induction of ER stress and of the 'unfolded protein response', which potentially results in the induction of caspase-dependent cell death involving mitochondria (71, 133, 135, 136, 178). It should be noted that all these mechanisms are intrinsically related to increased intracellular ROS generation (Fig. 3).

A first redox-dependent mechanism involved in insulin resistance is based on the activation of c-jun N-terminal kinase (JNK). Both TNF and FFAs are powerful modulators of the activity of two kinases, namely JNK and IκB kinase (IKK), which couple inflammatory and metabolic signals (133, 178). Interaction of TNF with its receptor results in activation of NADPH oxidase, mitochondrial outer membrane depolarization, and FFA-related mitochondrial dysfunction, ER stress

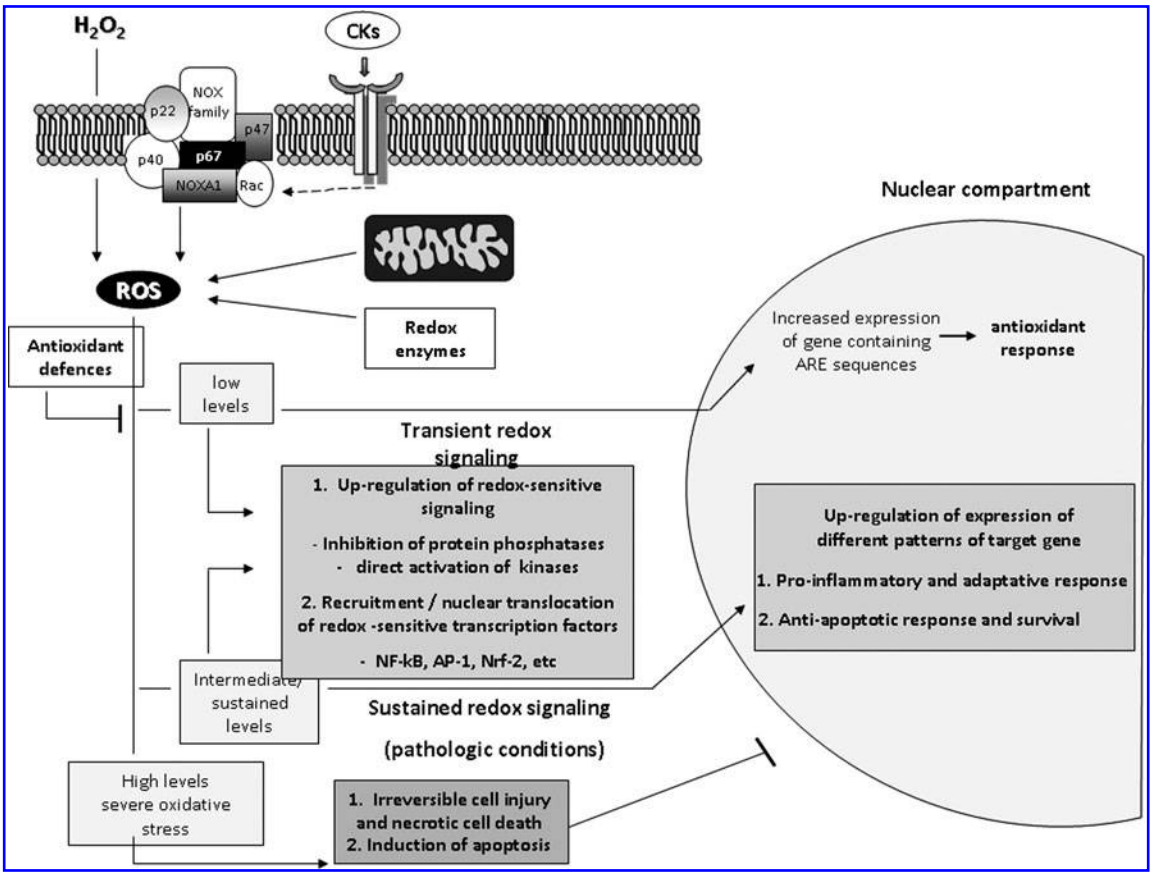

FIG. 4. Simplified scheme of redox signaling induced by increase in intracellular levels of ROS. Under physiological conditions, cells and tissues of aerobic organisms have to face relatively low amount (steady-state levels) of ROS, free radicals, and other reactive intermediates that are the result of a dynamic balance between the rate of their generation and the rate of their removal by one or more of defensive antioxidant systems (see Fig. 5), including highly specialized enzymes (catalase, thioredoxins, SODs, and GPXs), naturally occurring antioxidants (GSH, vitamin E, β-carotene, ascorbate, urate, and many others) as well as by amino acids, peptides, and proteins. Under these conditions, there is no significant unbalance of pro-oxidants vs. antioxidant defenses and thus no response by means of a redox signaling.

Whenever redox homeostasis is significantly disturbed, by an increase in ROS generation (whatever the source, intra- or extracellular), by a decrease in one or more antioxidants, or by a change in the thiol/disulfide redox state, redox signaling can be elicited. This can potentially lead to at least three different scenarios in which the difference is made primarily by the absolute levels of ROS and other reactive species reached within the cell as well as by the temporal length of the alteration. If the starting stimulus/condition is able to induce a relatively low and transient (*i.e.*, time limited) increase in intracellular levels of ROS and other reactive mediators, then also the shift in redox balance will be limited and redox signaling will operate through defined redox-sensitive signaling pathways and transcription factors to upregulate mainly transcription of genes able to encode for products that will reset in the due time redox homeostasis (for example, antioxidant enzymes, Trxs and Glrxs, cystine transport system to sustain genesis of GSH).

However, in conditions of extensive acute tissue injury as well as in tissues undergoing persisting injury, chronic inflammation, and chronic wound healing, levels of ROS and other related reactive intermediates or reactions (produced within the "target cells" or by extracellular sources, such as by inflammatory or damaged cells) may be very high and/or persistently increased within the cell to overcome antioxidant defenses and/or antioxidant response. If levels of ROS and related reactive intermediates are very high, this will lead to irreversible injury and necrotic—or apoptotic—type of cell death. Alternatively, when levels of oxidative stress are significantly high but not overtly able to induce irreversible cell damage, as in chronic inflammatory diseases, cells and/or tissues may still reach an altered equilibrium characterized by a shift of the intracellular redox state to higher levels of ROS and a chronically dysregulated state in which redox signalling is upregulating different patterns of gene expression and cell responses, then contributing to the progression of the disease.

and/or increased activity of cytochrome P450 isoforms like CYP2E1 and CYP3A (Fig. 2). On the other hand, ROS can activate JNK (177, 196) either through activation of the upstream apoptosis-stimulated kinase 1 (ASK) or inactivation of specific JNK-phosphatases (177, 196). Similarly, HNE has been shown to interact directly with JNK, leading to its activation (133). In the context of hepatic insulin resistance, activation of JNK, and particularly of JNK-1, leads to increased phosphorylation of IRS1 on serine 307, preventing its binding to the IR and propagation of insulin signaling (4, 73, 185). Upregulation of this inhibitory mechanism in association with insulin resistance should be considered as an exacerbation of a negative feed-back mechanism for the insulin signaling pathway (175), since insulin itself is able to activate JNK and

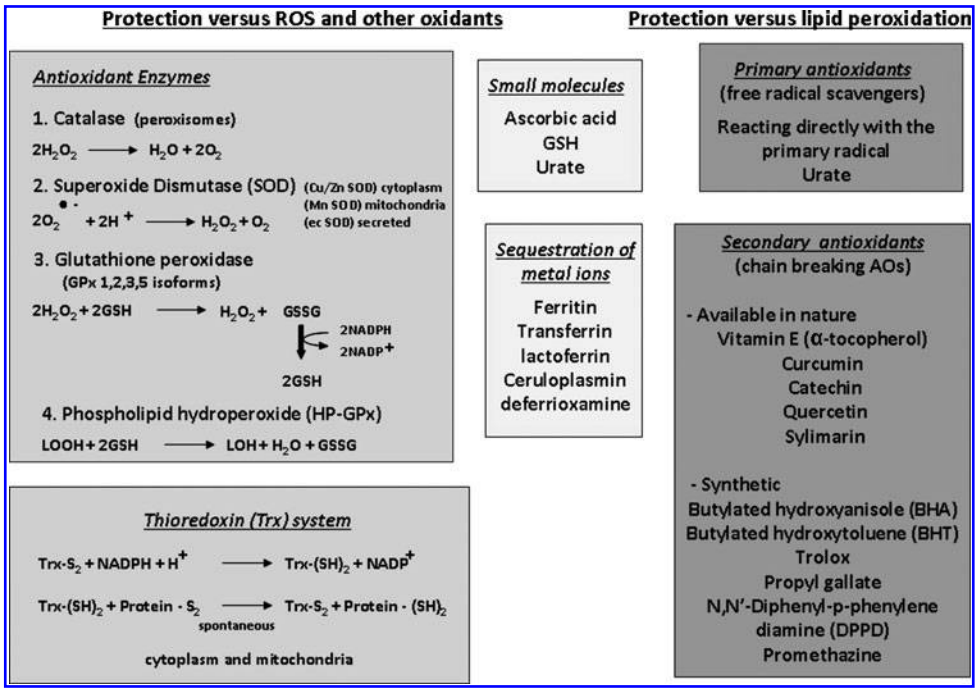

FIG. 5. Synopsis of major antioxidant defenses in a mammalian cell. Antioxidant defenses relies on the sum of those mechanisms that nature has developed to protect biological materials from ROS and other oxidants and those designed to protect them from lipid peroxidation. In order to simplify concepts, antioxidant defenses may be differentiated into two major (although inter-related) categories, separating defenses affording protection versus ROS and other oxidants from those mainly offering protection against lipid peroxidation. Within the first category we can include the following class of defenses:

1. *Antioxidant enzymes.* This definition includes a group of very specific enzymes such as catalase, glutathione peroxidase (GPX) isoforms, and superoxide dismutase (SOD) isoforms. Catalase and GPX isoforms are responsible for the removal of hydrogen peroxide but also of other organic hydroperoxides, whereas SOD isoforms operate by transforming superoxide into hydrogen peroxide. Major reactions of these enzymes are described.
2. *Small molecules.* This definition applies to several molecules but by far the most relevant are: a) ascorbic acid (vitamin C), a molecule that can act as an electron donor and then as a reducing agent; ascorbate can also scavenge (*i.e.*, interact directly with) •OH; b) *reduced glutathione* (GSH) that is a hydrosoluble tripeptide able to act as substrate for an enzyme able to remove H_2O_2 like GPX, as a scavenger of •OH and singlet oxygen, or as a low molecular weight thiol in regenerating oxidized –SH groups of proteins; in the figure, the essential reaction of GSSG reductase, designed to recover GSH, is also shown; c) *uric acid*, present in blood plasma, that has been reported to scavenge singlet oxygen, •OH, and peroxy radicals.
3. *Protection by sequestration of metal ions.* Transition metal ions such as iron and copper are able to lead to generation of very reactive species from less reactive ones. Then a number of metalloproteins such as ferritin, transferrin, metallothionein, and lactoferrin can be seen not only as relevant for their respective role in metal homeostasis but also as molecules that by "sequestering" redox active metal ions may prevent ROS production via the Fenton reaction.
4. *Thioredoxin and glutaredoxin systems.* Thioredoxins (Trxs), described in the figure, are small proteins that have a catalytic site containing two cysteine residues which can be oxidized reversibly to form disulfide bridges. They can undergo NADPH-dependent reduction by the enzyme thioredoxin reductase, and in turn they can reduce oxidized cysteine groups on proteins. These proteins, according to this intramolecular disulfide–thiol exchange, can act as hydrogen donors contributing to the control of redox state and redox signaling (for example, by affecting the regulation of kinases or transcription factors forming with them heterodimers).

Glutaredoxins (Glrxs) also belong to the thioredoxin superfamily of thiol/disulfide exchange proteins and in biological systems serve as reductants of protein–SG mixed disulfides and. similarly to what is described for thioredoxin system the glutaredoxin system (composed of Glrx isoforms, by glutathione reductase, GSH, and again NADPH) are also involved in redox signaling.

Where protection from lipid peroxidation is concerned, two classes of antioxidants can be described:

1. *Primary antioxidants,* also often defined as "free radical scavengers" because they are able to interact directly with, and/or to block the initiating free-radical (as for example •OH); examples are urate (able to scavenge peroxy- and alkoxy radicals as well as HOCl), and glucose (able to scavenge •OH with a rate constant similar to that of mannitol).
2. *Secondary or chain breaking antioxidants,* (α-tocopherol or vitamin E being the prototype) which are able to intercept radical intermediates produced during on-going lipid peroxidation such as peroxyl- or alkoxyl-radicals, then preventing (*i.e.*, "breaking") the perpetuation of hydrogen abstraction in the chain reaction.

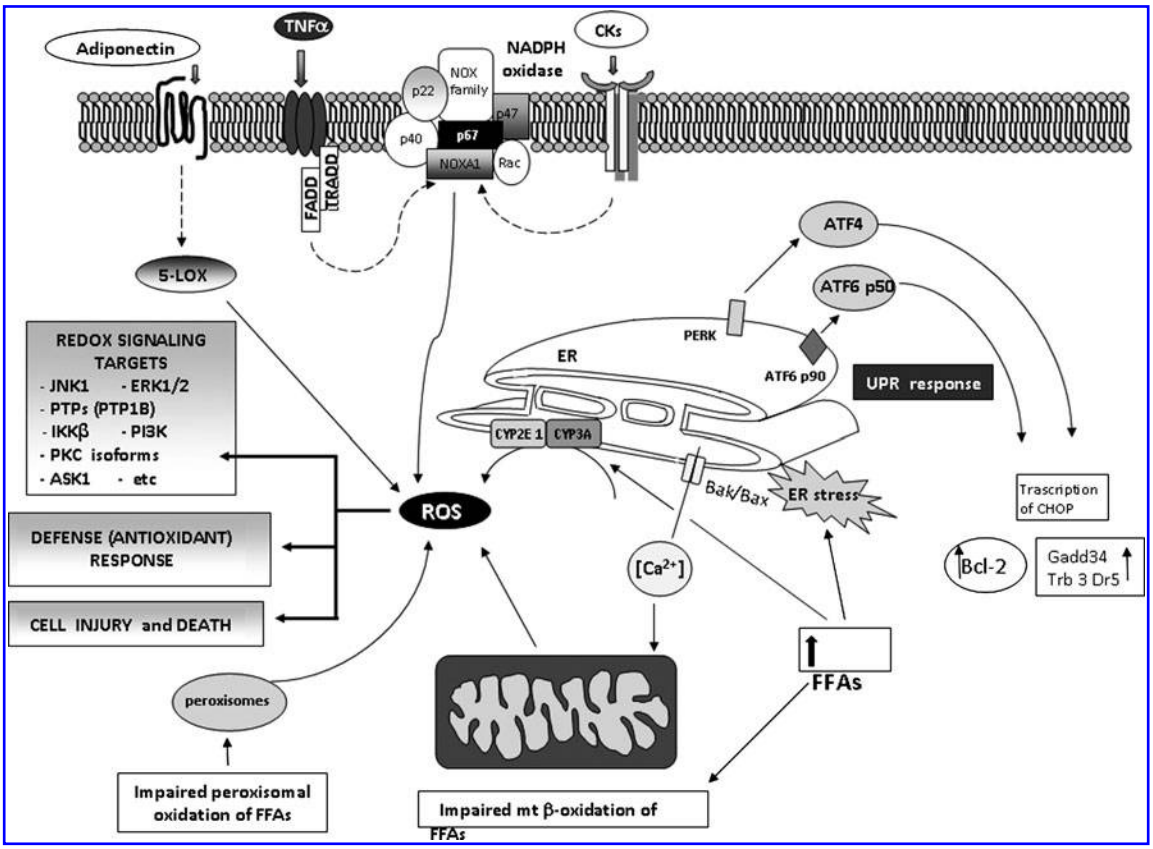

FIG. 6. Generation of intracellular reactive oxygen species and related consequences in fat-laden hepatocytes. In the peculiar hepatic milieu of a NAFLD/NASH patient, reactive oxygen species (ROS) can be generated within fat-laden hepatocytes by a number of mechanisms that, pertinent to this review, may include the following: 1) Activation of NADPH oxidase that is known to parallel interaction of several cytokines to their cognate receptors, including also TNFα; 2) activation of 5-LOX by adiponectin following interactions with its receptor; 3) impairment of mitochondrial β-oxidation of free fatty acids (FFAs); 4) impairment of peroxisomal oxidation of FFAs; 5) induction of endoplasmic reticulum (ER) stress by increased intracellular levels of FFAs and/or related upregulation of CYP2E1 and CYP3A isoforms of the cytochrome P450 family. The scheme, that also indicates major steps of FFAs induced, ER stress-related UPR response, recapitulates major consequences of increased intracellular levels of ROS. Depending on the actual levels of intracellular ROS, the cell response may be limited to increased redox signaling (as exerted by redox-dependent modulation of several signaling components) and upregulation of antioxidant defenses (*i.e.*, the so-called antioxidant response). Excess generation of intracellular ROS may lead to cell injury and death. More details can be found in the text.

other kinases which phosphorylate IRS on serine residues (see Fig. 1). However, to emphasize the multiple role of redox signaling, one should remember that in parallel, insulin has been shown also to activate NADPH oxidase, leading to a transient increase in ROS generation that through inhibition of PTP1B transiently enhances insulin signaling (112).

The redox sensitivity of JNK1 is likely to be critical: JNK activation not only prevents interaction of the IR with serine-phosphorylated IRS proteins but also results in caspase activation and induction of apoptosis (41). Moreover, JNK1 is likely also to control AP-1-dependent transcription, increasing the expression of proinflammatory cytokines and further contributing to JNK activation. JNK may have also a role in activation of the SREBP1 pathway, known to be involved in lipogenesis, which is increased in the presence of insulin resistance, while it should theoretically be decreased (64, 147). Several explanations have been proposed for this apparent paradox, including: a) the pathway leading to SREBP-1 activation may remain sensitive to insulin; b) the activation of lipogenesis by SREBP-1 and ChREB is sensitive to insulin as well as to high glucose levels; c) SREBP-1 can be activated by an insulin-independent mechanism related to ER-stress, that develops in liver steatosis (57, 83); d) SREBP-1 can be also activated by TNF as an additional contributory mechanism to increased hepatic lipogenesis (103).

Finally, JNK1 may have an additional role in regulating the expression and/or nuclear translocation of FOXO1, which is involved in gluconeogenesis. In this connection, insulin resistance results in a decrease of glycogen storage in hepatocytes in the postprandial state and in increased hepatic production of glucose (gluconeogenesis) in the fasting state (147). Data in experimental models indicate that fatty liver may directly induce hepatic insulin resistance by stimulating gluconeogenesis and activating PKC-ε and JNK-1, which may in turn impair the ability of insulin to activate glycogen synthase (144). Inhibition of hepatic glucose output by insulin involves Akt-dependent phosphorylation of FOXO1, a transcription factor controlling the expression of G6PC and

PEPCK (122, 130). Although NAFLD is characterized by hyperinsulinemia, under conditions of oxidative stress, as observed in NASH (43), FOXO1 becomes unresponsive to insulin because of interaction with the deacetylase sirtuin-1, resulting in induction of gluconeogenic genes (61, 131). The role of FOXO1 is supported by a recent study performed in control subjects and in patients with NAFLD or NASH (189), where it was found that: 1) expression of PEPCK is higher in patients with NASH than in those with simple NAFLD or normal liver, and was correlated with insulin resistance; 2) FOXO1 mRNA levels are higher in NASH and correlate with PEPCK; 3) in the presence of oxidative stress FOXO1 upregulation in steatohepatitis is associated with decreased Ser256 phosphorylation, with decreased Akt1 and increased JNK-1 activity.

Several kinases other than JNK-1 have been reported to be involved in insulin resistance as a possible consequence of increased generation of ROS and/or reactive nitrogen species (RNS) in response to a variety of stimuli, including hyperglycemia, elevated FFAs, or cytokines (52, 79). A critical example is represented by IκB kinase beta (IKKβ), a well known stress-sensitive kinase that controls the activation of NF-κB. Similar to other kinases, activated IKKβ phosphorylates IRS-1 on Ser307 residue, resulting in inhibition of insulin action (63). Interestingly, inhibitors of IKKβ such as salicylate, or ligands of peroxisome proliferator-activated receptor-γ (PPAR- γ) can restore insulin sensitivity (94, 208). Moreover, IKKβ (+/-) mice are more insulin-sensitive when compared to their control littermates (94, 208). In addition, treatment of a limited number of type 2 diabetes patients with high dose of aspirin resulted in reduced hepatic glucose production and fasting hyperglycemia as well as in increased insulin sensitivity (52).

Other kinases reported to be involved in oxidative stress-induced insulin resistance include p38 mitogen-activated protein kinase (p38MAPK), whose activation by oxidative stress inhibits insulin-stimulated glucose transport, as well as activation of mTOR, of several PKC isoforms (mainly PKCθ and PKCδ) and salt-inducible kinase 2 (SIK2) (reviewed in Ref. 52).

The action of adiponectin, a 'classical' adipokine, offers another example of a link between oxidative stress and insulin signaling. In the liver, adiponectin leads to AMPK phosphorylation that in turn causes decreased expression of genes involved in gluconeogenesis (*e.g.*, glucose-6-phosphatase, PECK) and lipogenesis (*e.g.*, SREBP-1) resulting in decreased hepatic glucose production and TG content. Adiponectin has also been reported to increase glycogen synthesis and aerobic consumption of glucose, and to exert an insulin-mimetic action that has been related to intracellular generation of ROS (58). Exposure of hepatocytes to globular adiponectin leads to a transient generation of ROS through activation of the small GTPase Rac1 and 5-lipoxygenase (5-LOX) (37, 133). This intracellular burst of ROS leads to ligand-independent transactivation of IR through oxidation and inactivation of PTP1B, a phosphotyrosine phosphatase controlling IR phosphorylation. In addition, ROS mediate the downstream response to both globular adiponectin and insulin, including activation of the MAPK cascade and ERK1/2 (58). However, insulin-mediated generation of ROS is related to NADPH-oxidase and not to 5-LOX. This form of redox signaling, based on reversible oxidation of PTPs, represents a strategy adopted by cells to reinforce receptor tyrosine kinase (RTK) signaling by avoiding inactivation by PTPs and has been described to parallel activation of many RTKs (37, 38, 133). These redox-related and insulin-mimetic mechanisms help to understand the *in vivo* effects of adiponectin on the liver (19, 212) and the muscle (33).

Adipokines, Oxidative Stress, and Inflammation in Fatty Liver

Several studies have addressed the impact of adipokines on the development of hepatocellular damage and inflammation, two major components of steatohepatitis. In most of these studies, a tight relation between adipokine expression and oxidative stress has been found. As circulating levels of adipokines have been shown to have an impact on the development of steatohepatitis, the relationships between oxidative stress and mechanisms of adipokine secretion in the adipose tissue are also briefly discussed.

Leptin

Leptin levels are usually elevated in obese patients, and have been found to correlate with systemic parameters of oxidative stress, suggesting that oxidative stress may contribute to adipokine imbalance in these subjects, especially in the presence of diabetes (164, 198). Leptin, like adiponectin, controls fat catabolism and glucose production activating central neural pathways and increasing hepatic AMPK activity (10, 137). However, the observation that steatosis is associated with elevated leptin levels in obesity indicates the presence of hepatic leptin resistance. The mechanisms of this disturbance are still poorly understood, and a role of nutrients, such as fructose, has recently been suggested (102, 140). Hyperleptinemia in fructose-fed rats is associated with high levels of tyrosine phosphorylation of STAT-3, but not of serine phosphorylation in nuclear STAT-3, suggesting a molecular mechanism for hepatic leptin resistance (140). Another mechanism leading to leptin resistance in this model has been related to increased levels of SOCS-3 and impaired phosphorylation on serine/threonine residues of proteins downstream of the leptin receptor, leading to reduced activation of FOXO1 and AMPK (191). Appearance of leptin resistance has also been linked to the endocannabinoid system, through activation of the CB1 receptor (134). Regulation of leptin receptors may be an additional component contributing to leptin resistance, as in diet-induced obesity, hepatic leptin receptor isoforms were found to be downregulated in the liver (25). Resistance to leptin should explain why obese patients with high leptin have fatty liver despite the anti-steatotic action of this adipokine.

Leptin is involved in innate and adaptive immunity. Survival and cytokine production by T-cells is promoted by leptin, which also stimulates phagocytic activity of macrophages, as well as chemotaxis of polymorphonuclear cells. As a consequence, ob/ob mice show reduced inflammation in autoimmune disease models but are more susceptible toward bacterial or viral infections. Leptin deficiency is associated with increased hepatotoxicity and mortality following endotoxin administration (205), an effect mediated by impaired macrophage function and cytokine imbalance (46). Conversely, leptin-deficient mice show less liver damage in models of T cell-mediated hepatitis, such as that induced by injection of concanavalin A, in association with lower levels of

TNF-α and IL-18 (55). Thus, leptin generally acts as a proinflammatory agent and participates in the protection from microbial infections. Additionally, leptin has a protecting role in models of alcoholic liver damage (13, 181). A possible mechanism underlying this action is the prevention of ethanol-elicited cytotoxicity and apoptosis, which was associated with decreased levels of reactive oxygen species (ROS) and an increase in antioxidant protective pathways (13). Conversely, leptin has also been involved in the generation of oxidative stress. Malondialdehyde levels in the liver of mice treated with a high fat diet were found to be directly related to circulating leptin levels, and leptin expression was upregulated in liver tissue (125).

Adiponectin

Oxidative stress is a relevant step regulating adiponectin secretion at the level of adipose tissue. In general, oxidative stress inhibits adiponectin secretion, linking adipose tissue inflammation, generation of reactive oxygen species, and downregulation of adiponectin secretion (62, 141). These data have been recently extended to children, where obesity-associated oxidative stress was inversely related to the levels of high-molecular weight adiponectin, the more metabolically active complex (11).

The mechanisms leading from adipose tissue expansion in obesity to oxidative stress and reduced adiponectin secretion are complex (Fig. 7). In rats, administration of angiotensin II results in hypertension and endothelial dysfunction, together with a decrease in adiponectin levels and in expression of adiponectin mRNA in the adipose tissue (68). These effects were inhibited by co-treatment with antioxidants, which also reduced angiotensin-mediated upregulation of NADPH oxidase. These results are relevant also to the pathogenesis of steatohepatitis and liver fibrosis, where the renin-angiotensin system plays a pivotal role (15). Interestingly, hydrogen peroxide was found to inhibit adiponectin expression in cultured adipocytes, confirming the negative modulation by oxidative stress (68, 89). The mechanisms underlying inhibition of adiponectin expression by ROS were further investigated in relation to the activity of uncoupling protein-2, which induces adiponectin, while inhibitors of mitochondrial respiration suppress it in a ROS-dependent fashion (36). The inhibition of adiponectin expression by ROS was mediated by upregulation of CHOP-10, which interferes with binding of C/EBPβ to the adiponectin promoter.

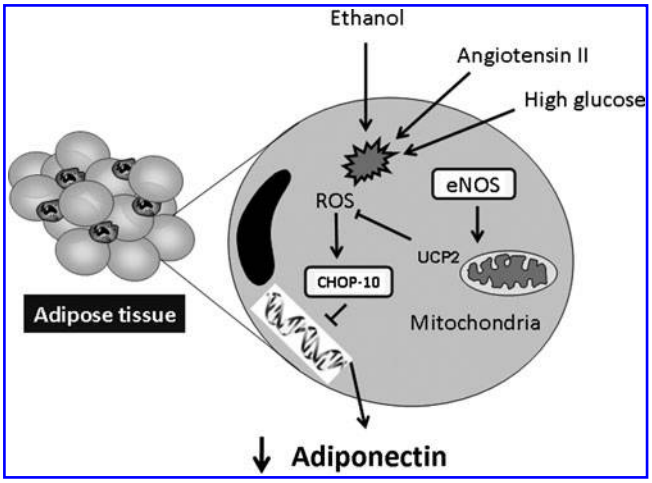

FIG. 7. Mechanisms of reduced adiponectin secretion involving oxidative stress. Generation of reactive oxygen species (ROS) within the adipocyte leads to reduced gene expression and secretion of adiponectin. Increased ROS production may be triggered by ethanol consumption, angiotensin II, or high glucose levels. In contrast, mitochondrial function reduces oxidative stress, partly due to the action of uncoupling protein 2 (UCP2). Reduction of ROS production is favored by eNOS expression, via production of NO, which increases mitochondrial biogenesis. ROS act on adiponectin gene expression by increasing the abundance of CHOP-10, which forms a complex with C/EBPβ, preventing its binding to the adiponectin promoter.

High glucose levels may be another factor leading to adipokine imbalance, including reduced adiponectin expression. Exposure of 3T3-L1 adipocytes to constant or intermittent high glucose suppressed the expression of adiponectin, and increased that of resistin in mature adipocytes, compared to normal glucose conditions. These actions were accompanied by increased levels of oxidative stress-related products and nitrotyrosine and were reverted by antioxidants (170). Along these lines, derangements in mitochondrial function have been identified as an additional mechanism relevant to adiponectin expression in the adipose tissue. Adiponectin expression and mitochondrial content in adipose tissue were reduced in obese db/db mice, while in cultured adipocytes, impairment in mitochondrial function decreased adiponectin synthesis (98). More recently, the same group demonstrated that endothelial NO synthase (eNOS) plays an important role in adiponectin synthesis by producing NO and enhancing mitochondrial function in adipocytes (97). Plasma adiponectin concentrations were reduced in eNOS knock-out mice, and this was associated with decreased expression of mitochondrial biogenesis factors, and increased levels of 8-hydroxyguanosine, a biomarker of oxidative stress. NO played a pivotal role, as chronic administration of a NO donor to eNOS-deficient mice increased both plasma adiponectin and adiponectin expression in adipose tissue. Generation of oxidative stress may even overshadow the beneficial effects of weight loss on adiponectin secretion. In subjects with acute weight reduction, increased urinary excretion of molecules related to oxidative stress was accompanied by a decrease in serum adiponectin (203).

Importantly for the pathogenesis of liver injury, ethanol has been shown to reduce the expression of adiponectin in the adipose tissue (34). The decrease in circulating adiponectin caused by chronic alcohol exposure was associated with increased homocysteine, while betaine reduced homocysteine and improved adiponectin (163). Accordingly, supplementation with taurine in a rat model of alcoholic liver injury resulted in normalization of serum levels of adiponectin and of its gene expression at the adipose tissue level, and in reduction of fat accumulation in the liver (35). As a mechanism, taurine prevented the decrease in C/EBP-alpha and PPAR-alpha, which regulate adiponectin expression, in response to ethanol administration. Similarly, treatment with mulberry leaf, which blocks experimental atherosclerosis, was recently shown to increase the expression of adiponectin, and to decrease that of TNF-α, MCP-1, and macrophage markers in white adipose tissue (169). These actions were associated with

reduced expression of NADPH oxidase in both adipose tissue and the liver. In ethanol-induced steatosis, resveratrol, a polyphenol with antioxidant properties, is another factor that limits fat accumulation and leads to increased adiponectin secretion (6). Nonetheless, in some models, adiponectin release may be positively regulated by oxidative stress. For example, downregulation of aldehyde oxidase-1 was associated with reduced adiponectin secretion (194).

Activation of PPAR-γ has emerged as an additional pivotal regulator of adiponectin expression and action. Thiazolidinediones, synthetic PPAR-γ ligands with antidiabetic effects, increase adiponectin levels in humans and induce its expression in experimental models (111). The tissue protective effects of thiazolidinediones are linked to both adiponectin induction and protection from oxidative stress, as exemplified in a model of ischemic heart disease (176). Targeted deletion of PPAR-γ in the adipose tissue, and studies on the adiponectin promoter sequence have confirmed the critical role of this transcription factor in the induction of adiponectin expression (69) (81).

More recently, additional data have indicated that part of the metabolic actions of thiazolidinediones may be mediated through PPAR-γ-dependent modulation of adiponectin receptor expression in different tissues, including the liver. In HepG2 cells, Sun *et al.* showed that rosiglitazone, a thiazolidinedione, increases the mRNA and protein levels of AdipoR2, the predominant adiponectin receptor in hepatocytes (171). These actions were confirmed by *in vivo* studies, where the hepatic levels of AdipoR2 were increased in mice treated with rosiglitazone. Along these lines, modulation of adiponectin receptor expression may have a relevant impact in the pathogenesis of both alcoholic and nonalcoholic steatohepatitis. In a mouse model of alcoholic hepatic steatosis, administration of rosiglitazone resulted in increased circulating levels of adiponectin and upregulated expression of both AdipoR1 and AdipoR2 in the liver (158). Remarkably, these changes were associated with activation of the sirtuin 1–AMPK pathway, and resulted in an amelioration of steatosis. Similarly, in rats administered a high-fat diet, as a model of nonalcoholic fatty liver disease, rosiglitazone improved histology and increased the expression of AdipoR1 and AdipoR2 in liver and visceral fat (109).

Adiponectin has in general a hepatoprotective and antifibrogenic effect in the liver wound healing process. In experimental steatohepatitis, administration of adiponectin ameliorates liver hepatomegaly, steatosis and necro-inflammation, through induction of hepatic fatty acid oxidation and inhibition of fatty acid synthesis (199). Accordingly, in obese mice subjected to damage by galactosamine/LPS, adiponectin administration protected from injury, reducing TNF and increasing PPAR-α (120). In addition, consumption of diet rich in saturated fat, which protects from alcoholic liver damage, increases adiponectin secretion (207). Although adiponectin and leptin share their effects as factors counteracting ectopic fat deposition, they have divergent effects on inflammation. In fact, while leptin-deficient mice are protected from T cell-mediated hepatitis, lipodystrophic mice that lack both adiponectin and leptin are not, unless adiponectin is administered (155). In general, adiponectin reduces inflammation, stimulating secretion of anti-inflammatory cytokines (*e.g.*, IL-10), blocking NF-κB activation, and inhibiting release of TNF-α, IL-6, and chemokines (179). Conversely, inflammation blocks adiponectin secretion, and specifically, adipose tissue inflammation contributes to reduce plasma adiponectin levels in obesity. Adiponectin protects also against Fas-mediated hepatocyte death (193). Therefore, adiponectin may be envisioned as a negative modulator of systemic and hepatic inflammation that characterizes the metabolic syndrome (66). Nonetheless, hepatic damage triggered by lipotoxicity may occur in spite of increases in adiponectin levels. In mice with steatohepatitis, antisense oligonucleotides interfering with diacylglycerol acyltransferase 2 decreased hepatic steatosis, but increased hepatic free fatty acids, lipid peroxidation, necroinflammation, and fibrosis. Progression of liver damage occurred despite reduced hepatic expression of tumor necrosis factor α and increased serum adiponectin (201). A similar dissociation between steatosis and steatohepatitis has been shown in a recent study where a diet deficient in methionine and choline was administered to mice defective in thioredoxin-binding protein-2 (TBP-2), an endogenous negative regulator of the antioxidant molecule, thioredoxin. These mice showed severe simple steatosis rather than steatohepatitis, and oxidative stress inflammation and hepatic fibrosis were attenuated in TBP-2(-/-) mice (5). These studies clearly indicate that fatty infiltration and steatohepatitis may be dissociated, and only the 'inflammatory' forms may progress to fibrosis.

Adiponectin modulates generation of oxidative stress-related products (Fig. 8). Initial evidence was provided by Yamauchi *et al.*, who cloned the AdipoR1 and R2 receptors and showed their ability to downregulate oxidative stress in the liver and adipose tissue (202). The molecular mechanisms by which this action of adiponectin is exerted are still unclear. In fatty liver, adiponectin downregulated hepatic expression of the enzyme aldehyde oxidase 1, which detoxifies aldehydes and generates oxidative stress (132). This effect was dependent, at least in part, on upregulation of PPAR-α, thus providing a link between the metabolic actions of this adipokine and regulation of the oxidative balance. In skeletal muscle, adiponectin was found to induce a number of NF-κB related genes, including ferritin heavy chain, which together with manganese superoxide dismutase is responsible for the protection from oxidative stress mediated by this adipokine (76). The antioxidant effects of adiponectin have been recently linked also to activation of AdipoR1 and the resulting downstream release of intracellular calcium together with increased activation of AMPK and sirtuin-1 (80). In macrophages, adiponectin modulates the response to lipopolysaccharide dependent on toll-like receptor-4, which leads to TNF-alpha secretion and generation of reactive oxygen species (75). Interestingly, this effect, which is mediated by expression of interleukin-10, occurs after an initial phase during which adiponectin actually increases TNF-α expression, indicating a complex action of adiponectin on the pathways regulating inflammation. Along these lines, in steatotic liver undergoing ischemia-reperfusion, adiponectin siRNA confer protection against oxidative stress and injury (121).

In line with these results, increase in hepatic steatosis and aminotransferase was observed in mice deficient for adiponectin, together with increase in thiobarbituric acid reactive substances, decrease in glutathione levels and expression of antioxidant enzymes (53). It is still unclear to what extent AMPK activation participates in the antioxidant action of

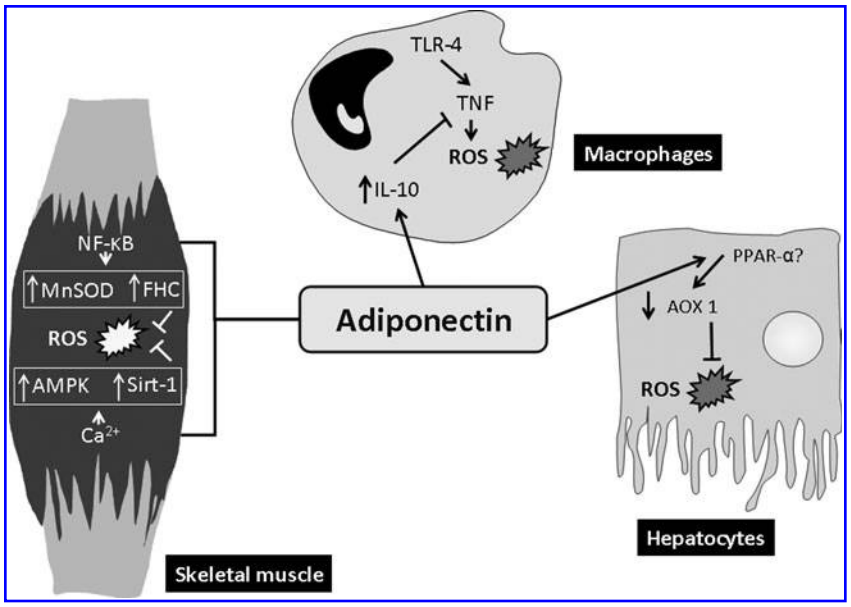

FIG. 8. Adiponectin reduces oxidative stress in different cellular targets. In hepatocytes, binding of adiponectin to its cognate receptors leads to activation of aldehyde oxidase-1 (AOX-1), which reduces intracellular levels of reactive oxygen species (ROS), possibly through increased activation of peroxisome proliferator-activated receptor (PPAR)-α. In macrophages, adiponectin increases the expression of the anti-inflammatory cytokine, interleukin-10 (IL-10). IL-10 blocks the generation of tumor necrosis factor (TNF) in response to activation of toll-like receptor-4 (TLR-4) by lipopolysaccharide. In skeletal muscle, adiponectin activates nuclear factor-κB that results in increased expression of the ferritin heavy chain (FHC) and of manganese superoxide dismutase (MnSOD). In addition, activation of AdipoR1 generates an increase in intracellular calcium that in turn is responsible of activation of AMP-activated protein kinase (AMPK) and of sirtuin-1. All these modifications result in reduced accumulation of ROS.

adiponectin in steatohepatitis. A study recently performed in a model of ischemia-reperfusion myocardial injury suggests that AMPK does indeed participate in protection against ischemia/reperfusion injury, but adiponectin still provides cardioprotection even after interference with AMPK activation (192). More important, treatment of mice defective in AMPK with adiponectin reduced oxidative stress to the same extent as in WT mice. In addition, it has been reported very recently in the same model that in mice made diabetic by administration of a high fat diet, the ability of adiponectin to activate AMPK is blunted, thus providing an additional mechanism for reduced cardioprotection and limitation of oxidative stress in diabetes (206). It still remains to be determined to what extent these data are applicable in the context of steatohepatitis.

Resistin

A limited number of studies have investigated the relevance of resistin in the pathophysiology of NAFLD. Improvement or worsening of hepatic insulin resistance was observed by reducing or increasing resistin levels, respectively (128, 146). The effects on the liver are also mediated centrally, as administration of resistin into the third cerebral ventricle stimulates glucose production independent of circulating levels of glucose-controlling hormones (127). Reduced hepatic glucose production in the absence of resistin is also associated with higher hepatic AMPK activation (14). Hepatic steatosis and VLDL secretion are decreased in resistin-deficient mice placed on a high-fat diet, suggesting a role of resistin in the induction of steatosis (160).

Additional studies link the biology of resistin with hepatic inflammation. In rats, resistin administration significantly worsens inflammation induced by LPS injection (16). In humans, resistin is expressed within the liver during severe damage, including the one associated with NASH, and correlates with infiltration of inflammatory cells (22). In rats, resistin administration significantly worsens inflammation after LPS injection, through the involvement of the coagulation cascade (16). In addition, in a model of cirrhosis, higher gene and protein expression of resistin and TNF-α was observed in epididymal fat, and TNF injection upregulated resistin (107). Expression of resistin has been documented in quiescent HSC, while activated human HSC respond to resistin with increased expression of pro-inflammatory chemokines and NF-κB activation (22, 157). Resistin expression has been linked to adipose tissue oxidative stress in humans (161). Support to this hypothesis is also provided by the recent observation that uremia increases the expression of resistin and retinol binding protein 4 via increased generation of oxidative stress in the adipose tissue and in isolated adipocytes (42).

Other adipokines

Adipose tissue is a source of a high number of factors, some of which are produced at high levels in other tissues, including the liver. The role of many of these factors in the pathogenesis of insulin resistance and in the development of steatosis and steatohepatitis has been the focus of recent reviews. In light of the limited data on oxidative stress, or for the presence of recent and comprehensive reviews, only a brief mention of the role of these molecules will be made herein.

TNF-α is a well-known pro-inflammatory cytokine that is abundantly released by adipose tissue. In addition to what described in previous sections of the present review, the action of TNF-α is intimately related to the upregulation of intracellular generation of ROS and then of oxidative stress (72, 133, 177–179). Hotamsligil *et al.* linked for the first time TNF-α to obesity, insulin resistance, and chronic inflammation (74), describing described significant elevation of TNF-α in adipose tissue of *db/db* mice. Following that seminal study, several other reports have progressively increased our knowledge on the role of TNF-α in relation to adipose tissue and hepatic NAFLD/NASH. In aggregate, the data summarized below suggest that TNF-α plays important roles in

the progression of NAFLD, including hepatic inflammation and fibrogenesis.

1. Adipose tissue of obese individuals is characterized by increased infiltration of macrophages and so-called "hypertrophied" adipocytes (195), the latter being able to release large quantities of free fatty acids (FFA) via macrophage-induced adipocyte lipolysis. FFA can serve as naturally occurring ligands for Toll-like receptor-4 (167, 168), and FFA increase the production of TNF-α in macrophages through a TLR4/ NFκB pathway, thus establishing a vicious cycle.
2. In the liver, Kupffer cells are the main producer of TNF-α, in a LPS-induced manner. In animal models, activation of Kupffer cells leads to induction of the TNF-α/ TNF receptor signaling pathway, which, as mentioned in the previous section, is critically involved in the pathogenesis of liver fibrosis in NASH (183). Along these lines, *ob/ob* are known to overexpress TNF-α (106) and indeed in these animals, treatment with anti-TNF antibody results in a significant improvement of NAFLD as a consequence of two major events: a) inhibition of c-Jun N-terminal kinase activity (involved in promoting insulin resistance), and b) decreased DNA binding activity of NFκB (involved in the acceleration/ amplification of inflammatory response).
3. In humans, TNF-α levels are significantly increased in both NAFLD and NASH. Moreover, TNF-α levels correlated with hepatic fibrosis in NASH patients (82), with gene expression of both TNF-α and of its receptor being significantly elevated both in hepatic and adipose tissues of human NASH patients (40). Accordingly, serum TNF-α levels have been confirmed to significantly correlate with NAFLD activity score (NAS) (114), a histologic scoring system that is becoming a standard reference in the grading of inflammation and damage in NAFLD. Along these lines, polymorphisms in the TNF-α promoter region and serum level of soluble TNF receptor 2 have been shown to correlate significantly with progression of NAFLD in human patients (180). Finally, the administration of the TNF-α inhibitor pentoxifylline was found to improve amino-transferase serum levels and the insulin resistance index assessed by homeostatic metabolic assessment (HOMA-IR) in NASH patients (2, 145).

Plasminogen activator inhibitors (PAI) decrease fibrinolytic activity by acting as rapid inhibitors of both tissue plasminogen activator (tPA) and urokinase-type plasminogen activator. Both the liver and the adipose tissue are major contributors to production of PAI-1 in humans (54). Insulin, very low-density lipoprotein, and free fatty acids induce PAI-1 production by the liver. In addition, PAI-1 is expressed by HSC and contributes to fibrogenesis (211).

PAI-1 expression in adipose tissue is upregulated in obesity (50), and hyperinsulinemia leads to increased PAI-1 plasma levels and gene expression (142). In addition, modulators of inflammation contribute to upregulation of PAI-1, which is an acute-phase protein. TNF-α, a pro-inflammatory cytokine overexpressed in adipose tissue, is another critical regulator of PAI-1 production (143). Different studies have demonstrated the presence of hypoxia in adipose tissue in conditions of obesity (47), and hypoxia leads to increate secretion of PAI-1 and decreased adiponectin secretion by adipocytes (70).

Several lines of evidence link generation of oxidative stress and expression of PAI-1 in adipose tissue and different tissues that develop chronic damage (8, 47). In adipocytes, high glucose and advanced glycation end products upregulated PAI-1 expression by oxidative stress-dependent pathways (186). Moreover, oxidative stress and PAI-1 secretion were inhibited by blockers of the renin-angiotensin system, a well-known mediator of adipokine imbalance (101).

In the liver, alcohol-related damage may be prevented, at least in part, by interference with PAI-1 (12). Moreover, upregulation of PAI-1 expression by alcohol in endothelial cells is inhibited by limitation of oxidative stress (162). PAI-1 has also been implicated in fibrogenesis, and in murine embryo fibroblasts the induction of PAI-1 by TGF-*β*, a profibrogenic cytokine, was mediated by activation of NADPH oxidase 4 and the resulting generation of ROS (108). This pathway causes increased activation of JNK and p38MAPK due to inactivation of MAPK phosphatase 1.

Monocyte chemoattractant protein-1 (MCP-1) is a proinflammatory chemokine that regulates migration of monocytes and lymphocytes. Hepatic expression of MCP-1 is regulated in different conditions of liver injury, and studies in genetically modified animals have identified protective or detrimental roles of this chemokine in various experimental conditions (209). Hepatic MCP-1 expression is also increased in patients with NAFLD, where it correlates with liver fat. This has led to the hypothesis that MCP-1 may be a critical regulator of the changes that are associated with NAFLD and the metabolic syndrome (20). Experimentally, interference with CCR2, the main receptor targeted by MCP-1, reduces metabolic alterations and fatty infiltration of the liver in lipoatrophic mice or in a mouse model of type 2 diabetes (172, 204). At least part of these effects are mediated by recruitment of inflammatory cells to adipose tissue, as indicated by studies in which conditional deletion of MCP-1 in adipose tissue was obtained (90). MCP-1 derived from the adipose tissue has also been shown to directly induce accumulation of lipids in hepatocytes (39).

Several lines of evidence link oxidative stress and MCP-1 expression in adipose tissue. Besides regulating the expression of adiponectin (see above), the antioxidant mulberry leaf inhibits expression of MCP-1 in white adipose tissue (169). In addition, angiotensin receptor blockade was associated with lower MCP-1 expression and generation of oxidative stress (105).

In the liver, a direct link between MCP-1 expression and generation of oxidative stress has been recently demonstrated in an acute model of hepatic damage (209). Hepatic stellate cells are responsible for a significant proportion of MCP-1 expression in the liver, and also in this case oxidative stress has been shown to promote expression of this chemokine (118, 200). Importantly, MCP-1 may be profibrogenic not only through its proinflammatory actions, but also directly, targeting activated stellate cells (119).

In humans, visfatin is increased in association with type 2 diabetes and the metabolic syndrome (137). Recently, visfatin has been identified as a circulating nicotinamide phosphoribosyltransferase, which catalyzes formation of nicotinamide mononucleotide and may influence the function of sirtuins (126). Along these lines, oxidative stress was found to reduce the expression of both sirtuins and visfatin in a monocytic cell

line (44). A role of visfatin in inflammation has also been suggested, and in a group of patients with NAFLD, plasma concentrations of visfatin could predict the presence of portal inflammation (9).

Chemerin regulates glucose uptake in adipocytes and stimulates lipolysis (65), and its serum levels are related to body mass index and the metabolic syndrome (24). In patients with NAFLD, chemerin levels were found to be elevated and to correlate with the NASH activity score (100). Chemerin has been associated with the induction of inflammation (51), and its possible relation with oxidative stress deserves future evaluation.

Adipokines, Oxidative Stress, and HSC Biology

Several *in vitro* and *in vivo* studies demonstrate a profibrogenic role for leptin. Absence of leptin or leptin receptor signaling, as occurring in *ob/ob* mice, and *fa/fa* rats respectively, markedly reduces the development of fibrosis in different experimental models of liver injury, including thioacetamide intoxication, chronic CCl_4 administration, or experimental NASH (78, 104). Whereas deficiency of leptin reduces fibrogenesis, injection of recombinant leptin during acute or chronic liver injury upregulates fibrogenic pathways (77). Conversely, administration of a leptin antagonist, or its co-administration together with recombinant leptin in a model of thioacetamide-induced fibrosis, markedly improves survival (49).

Leptin modulates the biology of different cell types which participate in the liver response to injury, such as Kupffer cells, sinusoidal endothelial cells, and activated HSC. Leptin increases phagocytic activity and cytokine secretion by Kupffer cells and macrophages (46) and stimulates endothelial cells to proliferate and to produce reactive oxygen species (138). Several groups have shown that leptin exerts direct actions on HSC, which express functionally active leptin receptors (7, 149, 151). Leptin modulates genes regulating extracellular matrix deposition and degradation in HSC (31, 149, 173), and in addition, it modulates proliferation and survival, via activation of different intracellular signaling pathways (151), and contributes to amplify the inflammatory response, via NF-κB activation (7). More recently, it has been shown that leptin enhances phagocytosis of apoptotic bodies by HSC (84), an event associated with HSC activation and fibrosis progression (210).

Recent studies have also investigated the intracellular signaling pathways involved in leptin-induced HSC activation and the role of oxidative stress in this context (Fig. 9). As with other cytokine receptors, generation of oxidative stress-related molecules participates in propagation of intracellular signaling. Exposure of LX-2, an immortalized human hepatic stellate cell line, to leptin, increases the intracellular levels of H_2O_2 in a Jak2-mediated fashion, contributing to the upregulation of TIMP-1 and type I procollagen (29, 31). Moreover, leptin was found to repress the basal level of MMP-1 mRNA and its promoter activity, an action again dependent on Jak2-mediated, H_2O_2-dependent, activation of ERK1/2 and $p38^{MAPK}$ (30). An example of the relevance of leptin-induced oxidative stress for the biology of HSC is provided by the effects of curcumin, which was able to block signaling downstream of the ObRb receptor interfering with ROS generation (174).

More recently, De Minicis *et al*. (45) showed that NADPH oxidase is a crucial mediator of the proliferative, fibrogenic, and inflammatory actions of leptin in HSC. Pharmacologic or genetic inhibition of NADPH resulted in reduction of the ability of leptin to induce HSC proliferation and upregulation of fibrogenic and inflammatory molecules (45). In addition, other data provide evidence that exposure of rat HSC to leptin results in the inhibition of the expression and activity of peroxisome proliferator-activated receptor-γ (PPARγ) (213), which maintains HSC quiescence and reverses HSC transdifferentiation to myofibroblasts.

An additional action of leptin that is relevant to the fibrogenic process is the modulation of hepatic angiogenesis. Ac-

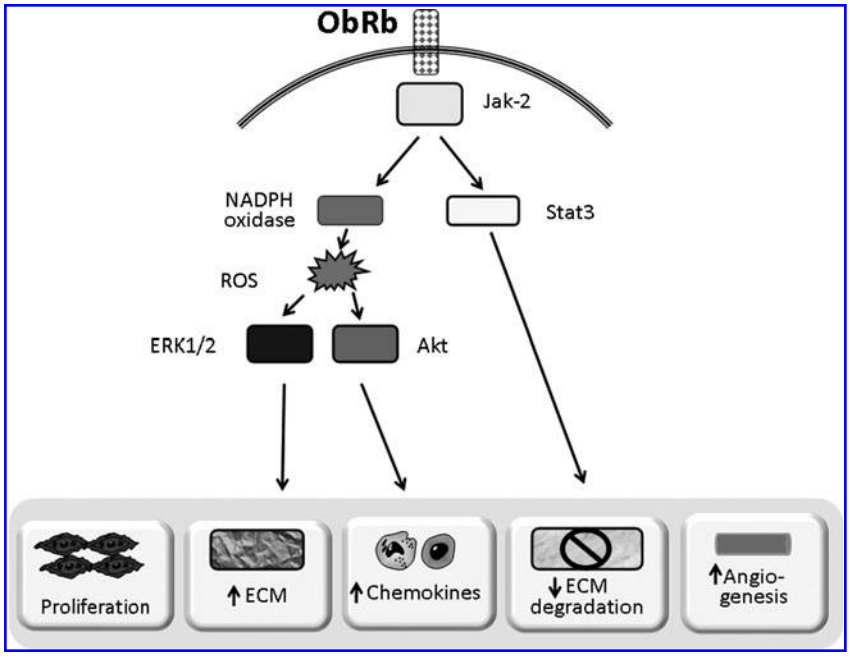

FIG. 9. Involvement of reactive oxygen species generation in leptin receptor signaling in hepatic stellate cells. Activation of the 'long form' of leptin receptor, ObRb, results in activation of the nonreceptor tyrosine kinase Jak-2, which activates Stat3 and NADPH oxidase, which leads to ROS generation. ROS contribute to the downstream activation of ERK-1/2 and of Akt. Stat3-dependent and independent signals control a number of biological actions that are pivotal for the actions of HSC in wound healing.

tivation of leptin receptors in HSC leads to upregulated expression of vascular endothelial growth factor, a potent inducer of neovessel formation (7). In addition, leptin exerts a direct angiogenic action on endothelial cells (159). Stimulation of neovessel formation in the liver by leptin is consistent with its profibrogenic role, as angiogenesis is a relevant component of chronic wound healing (190). Recent data indicate the involvement of oxidative stress also in this context (6a). On the other hand, the well-established association between angiogenesis and tumorigenesis suggests a possible additional role of leptin in liver cancer. Along these lines, recent data implicate leptin in the progression of hepatocellular carcinoma (HCC), as leptin increases growth, migration, and invasiveness of HCC cell lines (150). Additionally, leptin stimulates proliferation and metastatic potential of cholangiocarcinoma cells (56). In agreement with these data, lack of leptin is protective in a model of experimental steatohepatitis that leads to development of preneoplastic foci in the liver (96).

The ability of leptin to modulate biological actions through oxidative stress is not limited to the field of hepatic fibrogenesis, but has been involved in the development of atherosclerosis, another condition characterized by altered wound repair. In obese patients, phagocytic NADPH oxidase activity positively correlates with leptin and with carotid intima-media thickness, suggesting that hyperleptinemia may contribute to increased NADPH oxidase activity and early atherosclerosis (60). Leptin infusion in rats results in increased arterial pressure and endothelial dysfunction, two features which are relevant to the metabolic syndrome and to the pathogenesis of atherosclerosis. In aortic and renal tissue, the effects of leptin are mediated by elevation of oxidative stress-related products, including activation of NADPH oxidase (197). In addition, chronic hyperleptinemia reduces the expression of paraoxonase 1, an antioxidant enzyme contained in plasma lipoproteins (18). Of note, reduction of leptin-induced oxidative stress by antioxidant treatment also prevented the decrease in paraoxonase-1 (17). Along these lines, endothelial cells represent an additional target of the proatherogenic action of leptin, where oxidative stress may be involved. In these cells, leptin increased generation of ROS and activated JNK, resulting in increased expression of the proinflammatory chemokine, MCP-1 (23).

A direct antifibrotic effect of adiponectin has been demonstrated in rodents undergoing toxic liver damage, independent of the metabolic actions of this adipokine. Adiponectin knockout mice developed more extensive fibrosis after chronic CCl_4 intoxication, compared to wild-type animals (88). These effects are mediated at least in part by modulation of the activated phenotype of HSC, which express both adiponectin receptors (48). Full-length or globular adiponectin suppress multiple pro-fibrogenic actions of HSC (21), and activation of AMPK has been recently identified as a pivotal mechanism in this context (1, 27). AMPK activation occurs downstream of AdipoR1, but interference with AdipoR2 signaling has been recently shown to be sufficient to block the progression of experimental steatohepatitis (182). Adiponectin knockout mice develop more extensive fibrosis than wild-type animals after chronic CCl_4 intoxication, demonstrating that adiponectin has antifibrogenic effects independently of metabolic actions (88).

Appearance of hepatocellular carcinoma is a well-established consequence of fibrogenic liver diseases, and additional information has recently become available on the role of adiponectin in liver cancer. Administration of a choline-deficient, amino acid-defined diet to adiponectin-deficient mice resulted in increased incidence of liver tumors, together with increased levels of oxidative stress (87). In addition, lack of adiponectin was found to delay liver regeneration (53). In an orthotopic liver tumor model in nude mice, injection of adenovirus encoding adiponectin inhibited tumor growth, and was associated with lower appearance of distant metastases (113). These effects were accompanied by reduced activation of hepatic stellate cells and angiogenesis. Very recently, Saxena *et al*. (148) have shown that in hepatocellular carcinoma cells adiponectin increases apoptosis, inducing JNK phosphorylation. In addition, adiponectin-mediated increase in AMPK phosphorylation is a critical event for the induction of JNK phosphorylation and the biological effects of adiponectin. In parallel, knockout of JNK1 was found to protect from experimental steatohepatitis and to result in elevated adiponectin levels, thus demonstrating the complex role played by this mitogen-activated protein kinase in the context of adipokine biology and the pathogenesis of steatohepatitis (152).

Perspectives

The field of adipokines has witnessed a tremendous expansion in the last 5 years, and has led to the recognition of the role of adipose tissue and its products in several chronic diseases, including those affecting the liver. In addition, evidence for a cross-talk between adipokine biology, inflammation, oxidative stress, and tissue repair has been provided in several tissues in conditions of chronic damage.

In spite of the data that have accumulated, several points still await further clarification with experimental studies. In particular, the dual role of reactive oxygen species as intracellular signaling molecules and products accumulating during injury, make it difficult to reach general conclusions. Areas that will need additional investigation include: (a) elucidation of the role of oxidative stress in the context of chronic inflammation as a mediator of insulin resistance, as opposed to its action in the signaling cascade downstream of insulin receptor activation; (b) evaluation of the reasons why an approach with antioxidants, that is successful in animal models, has been still poorly validated in humans with insulin resistance and/or fatty liver; (c) detailed investigation of the role played by oxidative stress in mediating the response of the liver to adipokines, especially in the context of the angiogenic role of leptin and the anti-inflammatory and hepatoprotective role of adiponectin. As numerous laboratories are focused on the significance of this intriguing group of molecules, novel data are likely to be available very soon.

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Abbreviations Used

5-LOX = 5-lipoxygenase
AMPK = AMP-activated protein kinase
ChREBP = carbohydrate response element-binding protein
eNOS = endothelial NO synthase
FFA = free fatty acids
FOXO1 = forkhead box–containing protein O subfamily-1
GSK3 = glycogen-synthase kinase 3
HCC = hepatocellular carcinoma
HSC = hepatic stellate cells
IKK = IκB kinase
IR = insulin receptor
IRS = insulin receptor substrate
JNK = c-jun N-terminal kinase
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
MCP-1 = monocyte chemoattractant protein-1
mTOR = mammalian target of rapamycin
NAFLD = nonalcoholic fatty liver disease
NASH = nonalcoholic steatohepatitis
PEPCK = phosphoenolpyruvate carboxykinase
PI3K = phosphatidylinositol 3-kinase
PPAR = peroxisome proliferator-activated receptor
ROS = reactive oxygen species
RTK = receptor tyrosine kinase
SOCS = suppressor of cytokine signaling
SREBP-1 = sterol regulatory element binding protein-1
STAT = signal transducer and activator of transcription
TBP-2 = thioredoxin-binding protein-2
TG = triglycerides
TSC = tuberous sclerosis complex

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