4-Hydroxynonenal (HNE) and hepatic injury related to chronic oxidative stress

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4-Hydroxynonenal (HNE), a major end-product of free-radical activated peroxidation of polyunsaturated fatty acids, has attracted great scientific interest. HNE is more stable than free radicals and can diffuse within the cell or leave it and react with targets far from the initial site. These reactive aldehyde species are considerably reactive, producing multiple intra- and inter-molecular covalent adducts with biomolecules such as proteins, DNA and phospholipids. HNE is the most intensively studied aldehyde in relation to its physiological and protective function as a signalling molecule that stimulates gene expression and cell survival, as well as for its pathophysiological role as a toxic messenger that can propagate and amplify oxidative injury and promote mitochondrial dysfunction and cell death. Non-alcoholic fatty liver disease (NAFLD) is the most prevalent form of chronic liver disease in the world associated with oxidative stress, mitochondrial dysfunction and hepatocellular apoptosis. In this review we focus our attention on the molecular mechanism of the signalling and regulatory action of HNE. The role of HNE as a potent mediator for progression of liver injury in NAFLD is also discussed.
marker of oxidative stress but can act as a mediator of oxidative damage by modifying specific cellular functions [16]. Therefore, HNE is considered to be a toxic messenger that can deepen and expand oxidative damage in cells [8].

**HNE reactivity and metabolism**

HNE has high reactivity and interacts easily with thiol (-SH) or amino (-NH₂) groups of glutathione and proteins, and at high concentration, with DNA. HNE forms covalent adducts with proteins in the cell, including ones in the plasma membrane, with the modification of specific cellular functions [16,17]. Plasma membrane proteins represent the first targets for adduct formation. HNE can also interact with nuclear proteins, hence modifying protein expression via their interaction with transcription factors or other regulatory compounds [7,8]. Functional and or signalling proteins modulation probably represents one of the main mechanism by which HNE can modify both physiological and pathological processes [18] (Figure 1).

As recently reviewed [17], HNE-protein adducts are physiological products of metabolism which are sometimes elevated in tissues or plasma in diseases [1]. HNE protein adducts at low concentrations play an important role in cell signal transduction and gene expression including receptors, kinases, phosphatases and transcription factors [7,19,20].

The high reactivity that HNE has leads to an array of effects on various proteins, resulting in changes in their function and stability. High levels of HNE may influence cellular function and behaviour [17]. The formation of cross-linked HNE-protein adducts might notably affect the cellular senescence process and, thus, contribute to aging [3,17,20]. Studies have shown that the generation of HNE-protein adducts can play important pathogenic roles in age-related neurodegenerative diseases, atherosclerosis and cancer [21–24]. Many animal model studies and clinical trials have affirmed that HNE modified proteins are one of the important factors in the development and progression of chronic liver diseases [25,26].

The generation of HNE-protein adducts can represent a contrast to the progression of disease or can promote adaptive cell responses, demonstrating that HNE is not only a toxic product of LPO but also a regulatory molecule that is involved in several biochemical pathways [4,26]. While high concentrations of lipid peroxidation products such as 4-HNE cause cellular damage and apoptosis, in the low concentration range, HNE exerts adaptive cytoprotective effect. In other words, the effect of 4-HNE on the cell may be damaging or protective, depending on its concentration, respectively, not only on its production but also on the clearance of this molecule [2]. This suggests that, in some diseases, HNE may act as a target for detection, early prevention, and even cessation of disease progression [27].

The degradation of HNE is an important part of the antioxidant defense. HNE is degraded to non-toxic or less toxic compounds which are either metabolised or excreted [17]. The key HNE-degrading enzymes are glutathione S-transferases (GST), alcohol dehydrogenases

![Figure 1. Reactivity, metabolism and possible biological effects of 4-hydroxynonenal.](image-url)
(ADH) and aldehyde dehydrogenases (ALDH) [3,17,28]. In rat hepatocytes, more than half of HNE is metabolised to the GSH-HNE intermediate conjugate [17,29]. GSH-HNE and other intermediates are among the main determinants of the intracellular levels of HNE because they have the ability to modulate stress-induced signalling for programmed cell death. These processes, in turn, limit the apoptotic potential of HNE [2,20,30]. HNE detoxification by GSTs can be reduced due to depletion of GSH, which in turn may lead to increased levels of HNE [31,32]. On the other hand, S-adenosyl methionine (SAM) diminishes HNE modification of enzymes which have a vital role in the detoxification of HNE [32]. N-acetyl cysteine (NAC) is a precursor of GSH synthesis and a physiological HNE scavenger, which may directly neutralise HNE via its antioxidant and aldehyde scavenger properties [33].

HNE in signalling pathways

The most widely described roles of HNE in the signalling pathways are associated with its activation of kinases, as well as transcription factors that are responsible for redox homeostasis (Ref-1, Nrf2, p53, NFκB) [34]. Depending on its level, HNE exerts harmful or protective effects associated with the induction of antioxidant defense mechanisms [34].

Oxidative stress and lipid peroxide HNE may activate Nuclear Factor-κB (NF-κB) a redox-sensitive transcription factor that regulates expression of numerous genes and plays important roles in immune and stress responses, inflammation, and apoptosis [35,36]. NF-κB is sequestered in the cytoplasm of cells and is bound it inhibitor IkB. NF-κB is activated by degradation of inhibitor by HNE and inflammatory cytokines [3]. After liberation, NF-κB translocate to the nucleus binding to DNA and enhances the expression of genes TNF-α, IL-1β, IL-6 and IL-8 and consequently lead to mtDNA damage which results in generation of more reactive oxygen species (ROS), forming a vicious cycle of oxidative stress [37]. Therefore, antioxidants that inhibit the activation of NF-κB may be attractive strategy for limiting hepatic injury in NAFLD [38,39]. NF-κB and activator protein 1 (AP1) are downstream signalling molecules that couple receptor ligation to the activities of several classes of signal-dependent transcription factors, such as c-Jun NH2-terminal kinase (JNK) [40,41].

HNE could act as a potential activator of Nuclear factor erythroid 2-related factor 2 (Nrf2) [3,42,43]. The mechanism involves stress-generated electrophiles and oxidants that switch on the Nrf2 dependent cellular defense pathway. In this defense pathway, Nrf2 is released from Keap1 and translocated to the nucleus where it binds to the antioxidant response element (ARE) and initiates the transcription of antioxidant and protective genes [34,44,45]. Many antioxidant enzymes are regulated by Nrf2, including: NAD(P)H quinone reductase 1, which neutralises the toxicity of quinones; glutamate-cysteine ligase, which regulates GSH synthesis; sulfiredoxin one and thioredoxin reductase 1, which help to detoxify peroxides, hydrogen peroxide and peroxynitrite; heme oxygenase-1 (HO-1), which catalyses the degradation of heme; GST, the cystine/glutamate amino acid transporter and other protective systems [44,45]. Therefore, HNE activating Nrf2 can induce antioxidant defense mechanisms to restrain its own production and to enhance the cellular protection against oxidative stress [22,43]. The role of Nrf2 in the progression of NASH has been studied [46]. It was found that high fat diet induced an increase in lipid peroxidation and Nrf2 expression, which strongly correlated with the degree of hepatic steatosis and inflammation [47]. In mice with diet-stimulated nonalcoholic steatohepatitis, pharmacologic activation of transcription factor Nrf2 improves glucose homeostasis and inhibits hepatic steatosis, inflammation, and fibrosis. Nrf2-mediated amelioration of nonalcoholic steatohepatitis and liver fibrosis involves downregulation of lipogenic genes, induction of antioxidant genes, and suppression of both oxidative and endoplasmic reticulum stress [48].

HNE and apoptosis

The role of HNE in apoptosis has been reviewed more extensively previously [20]. Briefly, what makes HNE a special apoptotic inducer is that it forms protein adducts in mitochondria, causing mitochondrial dysfunction, and propagates oxidative injury [1,14,20,23]. HNE can directly induce apoptosis but it can also be a mediator of apoptosis, as it can be generated by ROS [14,20]. HNE can stimulate intrinsic and extrinsic apoptotic pathways and interact with typical actors such as JNK, Fas or mitochondrial regulators [6,12,15,20,22,49].

HNE induces the intrinsic apoptotic pathway. The intrinsic apoptotic pathway, also called the mitochondrial pathway, is the major apoptotic pathway [20]. ROS and lipid-peroxides products such as 4-HNE directly damage mitochondrial (mt) DNA and activate the mitochondrial apoptotic pathway. Oxidation of cardiolipin (the major lipid component of the inner mitochondrial membrane), which generates HNE, is necessary for the subsequent steps of intrinsic
apoptosis [50,51]. In addition, HNE oxidises the SH-groups (cysteine, methionine) of glutathione, which changes the redox status and is an early and potent activator of apoptotic signals [13,14,49,52,53]. According to Liu et al. [52], HNE-induced reduction of cellular GSH/GSSG pool is closely associated with the activity of HNE to induce apoptotic cell death via Fas-independent activation of caspase 3, possibly through a mitochondria-dependent pathway.

Mitochondria are very sensitive to the damaging effect of HNE. Damage to mtDNA by HNE causes defects in respiratory enzymes, decreases the capacity of reducing potentials and energy production. On the one hand, it further enhances the generation of ROS and reactive nitrogen species (RNS) in mitochondria, resulting in disturbance in antioxidant defense [8,53]. This disruption acts as an apoptotic signal [20]. Under the effect of an apoptotic signal (mtDNA damage), the pro-apoptotic Bax proteins undergoing conformational changes, are translocated from the cytosol to the external mitochondrial membrane where the antiapoptotic Bcl-2 proteins are located. The pro-apoptotic protein Bax increases the permeability of the external mitochondrial membrane, which allows the release of cytochrome C and other pro-apoptotic molecules in the cytosol and activation of caspases and the mitochondrial pathway of apoptosis [54–56].

HNE has a significantly different role compared to other lipid oxidation products in the regulation of two essential anti-apoptotic elements, the NF-κB transcription factor and its target anti-apoptotic B-Cell Lymphoma-2 (Bcl-2) protein. There is evidence for presence of a HNE-based downregulation of Bcl-2 expression, mostly likely via NF-κB. The crosstalk of NF-κB pathway and Bcl-2 was proposed to be mediated by HNE [36].

**HNE induces the extrinsic apoptotic pathway.** The extrinsic apoptotic pathway transmits a death signal from the cell surface to the intracellular compartments and is initiated by the activation of death receptors like receptors of TNF-α, Fas/CD95 or TRAIL (TNF-related apoptosis-inducing ligand). The induction of Fas by HNE is associated with JNK activation and apoptosis induction [57]. JNK is known to have a central role in both intrinsic and extrinsic pathways. Activation of JNK pathways and production of reactive oxygen species (ROS) from fructose metabolism can stimulate the production of inflammatory mediators (via NF-κB) or induce direct apoptosis. On the other hand, apoptosis may also be activated by the external receptor-dependent pathway via Fas ligand, TNFR1 and TRALL receptors [58]. It has been shown that elevated serum TNF-α levels [3] and saturated CMKs can induce apoptosis by binding to the corresponding membrane receptors in a ‘death-inducing signal complex’ activating the catalytic activity of effector caspases [59,60]. HNE also triggers NF-κB activation, which then induces TNF-α synthesis and consequently mtDNA damage.

Stimulation of the death receptors by TNFα may also increase the expression of the pro-apoptotic Bax in the external mitochondrial membrane and the release of cytochrome C, thereby binding the death receptor and mitochondrial pathway [61,62].

The pro-apoptotic activity of HNE as a marker of lipid peroxidation and oxidative stress is associated with: (i) direct damage and mtDNA activation of the mitochondrial apoptotic pathway; (ii) inhibition of mitochondrial proteins (channels) such as adenine nucleotide translocase (ANT) due to SH-group oxidation, which increases the permeability of mitochondrial membrane for Cyt-c release; (iii) inhibition of antiapoptotic Bcl-2 [63].

**HNE and liver injury in NAFLD**

Diets rich in fat, as well as high-fructose diets, and high-cholesterol diets contribute to the pathogenesis of nonalcoholic liver disease (NAFLD) [64]. This disease constitutes an increasingly prevalent liver disorder and has been suggested to be the hepatic manifestation of the metabolic syndrome [17,65]. NAFLD is a clinical-pathological syndrome that encompasses a wide spectrum of liver damages, ranging from steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis in the absence of alcohol abuse [66]. The pathogenesis of this disease is multifactorial and not fully elucidated. Studies have shown that fat accumulation in liver cells, imbalance in adipocytokines secretion, oxidative stress and low-grade inflammation, are responsible for the development of mitochondrial dysfunction, stimulating hepatocyte apoptosis in the steatotic liver [67,68].

The mechanisms by which steatosis progresses to NASH are still not fully understood; however, growing evidence has suggested that oxidative stress is involved in this process [69–73]. Oxidative stress is generated through oxidation of free fatty acids, cytochrome P450, iron overload, and necro-inflammatory cytokines such as TNF-α [74–76]. Ensuing excessive ROS production enhances lipid peroxidation, which subsequently decreased the antioxidant defense, activates lipid peroxidation and elevates the intracellular and extracellular content of 4-HNE, and results in hepatocyte damage and liver injury [17,25]. However, the cellular and molecular mechanisms of HNE-induced
liver injury associated with chronic fructose and lipid consumption have not been well elucidated yet. Some of the most relevant recent findings of HNE as a bioactive marker of oxidative stress are summarised in Table 1.

Chronic fructose consumption causes fat accumulation in the liver and liver steatosis [77]. Surplus of fatty acids in fatty liver leads to mitochondrial production of free radicals which may stimulate inflammatory response and liver injury in NAFLD. Extra-mitochondrial reactions mediated by NADPH oxidase, xanthine oxidase and inducible nitric oxide synthase (iNOS) may contribute to the elevated ROS/RNS production in NAFLD [78,79]. Elevated oxidative stress has been well documented in NAFLD patients [17,73,80]. Excessive ROS production overwhelms antioxidant defenses and generates highly toxic HNE lipid peroxides. Significant increase in serum levels of HNE levels has been demonstrated in patients with NASH, compared to those with simple steatosis [81]. An excessive generation of HNE detected by antibodies, like anti-HNE protein, was demonstrated in liver in experimental models of NAFLD [84]. Thus, low levels of GSH and decreased expression and/or activity of some glutathione-related enzymes may have additional deleterious effects on fatty liver [52,80,85].

Mitochondrial dysfunction and oxidative stress play a central role in the pathophysiology of NAFLD/NASH [86]. Mitochondria are very sensitive to the damaging action of lipid peroxide HNF, which oxidise SH-groups (cysteine, methionine) of glutathione, change the redox status and are an early and powerful activator of apoptotic signals [70]. The formation of HNE adducts, GSH depletion and protein oxidation are major events associated with mitochondrial dysfunction, which represent critical initiating events for the development of NAFLD [83]. HNE is a special apoptotic inducer because of its abilities to oxidise mitochondrial DNA, protein and to form protein adducts [20]. A significant increase in mitochondrial HNE–protein adducts during NASH development has been also reported [68–72]. In addition, oxidation of biomolecules by mitochondrial ROS initiates a vicious cycle of exacerbated mitochondrial dysfunction and cell death [79].

HNE level is elevated in steatosis liver, indicating oxidative damage of mitochondria. In rats with fructose-induced metabolic syndrome the expression of pro-apoptotic BAX in the liver is increased, whereas that of Bcl-2 decreased. Similar findings for apoptotic proteins Bax and Bcl-2 in SECs and hepatocytes are

| Findings | Type of study/methods | References |
|----------|----------------------|------------|
| 4-Hydroxynonenal (HNE) is lipid peroxidation product exerts a multitude of biological, cytotoxic, and signal effects | In vivo and in vitro studies | Dianzani [1]; Yang et al. [31]; Zhang et al. [44]; Awasthi et al. [6]; Chaudhary et al. [49]; Ott et al. [53]; Le Bras et al. [54]; Timucin and Basaga [36] |
| HNE form protein adducts in mitochondria, mitochondrial dysfunction and induce apoptosis | Animal and clinical studies, histochemistry, immunohistochrometry, structural and enzymatic analysis | Dalleau et al. [20]; Shoeb et al. [21]; Sala et al. [22]; Di Domenico et al. [23] |
| HNE-protein adducts play important pathogenic roles in age-related neurodegenerative diseases, atherosclerosis and cancer | Clinical and animals studies, apoptosis, immunohistochrometry using genuine polyclonal or monoclonal antibodies | Poli et al. [4]; Castro et al. [17] |
| HNE is a marker of liver damage and one of the causative agents in chronic liver diseases | Animal and clinical studies, histochemistry, immunohistochrometry, structural and enzymatic analysis | Bellanti et al. [72]; Seki et al. [69]; Servidio et al. [71] |
| HNE may be an essential factor of non-alcoholic liver disease | Clinical studies, histochemistry, immunohistochrometry, structural and enzymatic analysis | Malhi and Gores [75]; Schreuder et al. [76]; Sumida et al. [33]; Rolo et al. [70] |
| HNE modified proteins are one of the crucial factors in development and progression of chronic liver diseases | Animal and clinical studies | Chen and Niki [2]; Timucin and Basaga [36]; Zhang et al. [44]; Łuczaj et al. [34]; Brown et al. [32] |
| HNE activates signalling pathways Nrf2, NFκB. Depending on its level, HNE exerts harmful or protective effects associated with the induction of antioxidant defense mechanisms | Animal and clinical studies | Ott et al. [23]; Perico et al. [44]; Timucin and Basaga [36]; Zhang et al. [44]; Łuczaj et al. [34] |
| HNE is a physiological constituent of various tissues | Mostly analysis of tissue homogenates, blood analysis usually using chromatography, recently also tissue immunohistochemistry | Di Domenico et al. [7] |
demonstrated in an experimental model of NAFLD [87]. Under physiological conditions Bcl-2 located in the mitochondrial membrane suppresses oxidative stress and prevents cell apoptosis [88]. The activation of oxidative stress by TNF-α and free fatty acids translocation of the BAX protein from the cytosol and its transfer to the external mitochondrial membrane result in leakage of cytochrome C and other pro-apoptotic proteins and activation of programmed cell death [14]. Depletion of mitochondrial GSH levels could also be due to its reduced uptake by mitochondria as a result of enhanced levels of cholesterol within the inner mitochondrial membrane and the decrease in synthesis of SAM, the major methyl donor in liver and precursor to GSH [89]. An increased HNE adduct level and GSH deficiency may promote the opening of mitochondrial permeability transition pores, which is a critical factor for cell death [90]. In mice with diet-stimulated nonalcoholic steatohepatitis, a pharmacologic activator of Nrf2 (Curcumin) prevents the NASH by mitochondria protection and apoptosis reduction and providing a possible novel treatment for NASH [25].

Additionally, lipoperoxide products such HNE increase the production of pro-inflammatory cytokines such as TNF-α, IL-6 and CRP, and contribute to apoptosis and progression of liver injury in NAFLD [91]. Bcl-2 and BAX protein, major mediators of apoptosis, are mainly activated in the internal (mitochondrial) signalling pathway. It has been reported that the stimulation of the death receptors by TNF-α may also increase the expression of the pro-apoptotic Bax in the external mitochondrial membrane and may result in release of cytochrome C, thereby binding the death receptor and mitochondrial pathway [61,62,92]. Lipid peroxide HNE in steatotic liver may synergistically activate the JNK/c-Jun signalling pathway that augments liver injury and apoptosis [93]. HNE-induced JNK activation is inhibited by pretreatment of the cells with a thiol antioxidant, N-acetylcysteine [41,72,94]. Tsuruta et al. [95] showed JNK-induced Bax translocation to mitochondria, cytochrome C release and apoptosis. Sustained activation of the c-Jun NH₂-terminal kinase (JNK) signalling pathway mediates the development and progression of experimental diet-induced NAFLD [95].

Data from a study by Malhi et al. [96] show that saturated fatty acids also induce JNK-dependent hepatocyte apoptosis by activating pro-apoptotic Bid-Bach proteins from the internal signalling pathway. Antioxidant gingolide A increased the levels of anti-apoptotic Bcl-2, while it decreased Bax, phosphorylated JNK and cleaved caspase-3 and -9 levels in the livers of animals with high fat diet [97]. The manifestation of a synergistic effect on the activation of the JNK signalling pathway from depletion of mitochondrial reduced glutathione and elevated peroxides, such as MDA, was found in the steatal liver in experimental animals and patients [74].

HNE is one of the major end-products of lipid peroxidation generated during the transition from simple steatosis to steatohepatitis [72]. Therefore HNE generated by NAFLD is not only a passive biomarker but rather an active mediator of hepatocellular injury [31,93]. Thus HNE may mediate the progression from steatosis to hepatocyte injury by direct oxidative modification of cellular molecules and also by activation of cell death signalling pathways such as JNK/c-Jun.

Conclusions

The increased evidence in liver tissue and plasma definitely supports a significant role of oxidative stress and free-radical-induced HNE production in liver pathophysiology. This aldehyde is not only a toxic byproduct of oxidative stress metabolism but, on the contrary, it is an important factor in the mechanism of cell signalling. Studies with NAFLD rats show pathological HNE features such as steatosis, dysregulated oxidative unbalance, low-grade inflammation, early mitochondrial dysfunction and apoptosis, which can be prevented in the presence of antioxidants.

Disclosure statement

The authors declare no conflict of interest.

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