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Review Article

4-Hydroxynonenal (HNE) modified proteins in metabolic diseases

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ABSTRACT

4-Hydroxynonenal (HNE) is one of the quantitatively most important products of lipid peroxidation. Due to its high toxicity it is quickly metabolized, however, a small share of HNE avoids enzymatic detoxification and reacts with biomolecules including proteins.

The formation of HNE-protein-adducts is one of the accompanying processes in oxidative stress or redox disbalance. The modification of proteins might occur at several amino acids side chains, leading to a variety of products and having effects on the protein function and fate.

This review summarizes current knowledge on the formation of HNE-modified proteins, their fate in mammalian cells and their potential role as a damaging agents during oxidative stress. Furthermore, the potential of HNE-modified proteins as biomarkers for several diseases are highlighted.

1. Introduction - proteins as targets for oxidative modifications

Metabolic disorders such as the metabolic syndrome, type 2 diabetes and cardiovascular diseases have been increasing over the years contributing to a decrease in both life and health span. Development of more powerful prevention approaches are, therefore, required and pass through understanding the molecular mechanisms adjacent to the development of such limiting diseases for human life´s quality. From a cellular and molecular perspective, which factors decisively contribute to metabolic disorders? Oxidative stress is thought to play a role in tissue dysfunction by disrupting cellular redox signaling and by impinging oxidative damage on biomolecules, resulted by the attack of reactive oxygen species (ROS), which accumulate over time. This has been widely proposed as the main cause of the aging process and moreover, to play a role in dysregulating metabolism. Oxidative stress has been described to be closely related to metabolic alterations such as obesity and metabolic syndrome [1], in multiple forms of insulin resistance [2] and in pancreatic β-cell death mediated by ROS [3]. Chronic oxidative stress results in increased levels of oxidized proteins which can be sufficient to trigger cellular dysfunction and cell death, since practically the whole metabolism relies on proteins to execute manifold cellular processes. Amongst the several modifications proteins can bear, protein carbonylation has been accepted, due to its irreversibility and stability, to be a relevant modification. Oxidative carbonylation is a non-enzymatic phenomenon which leads to protein dysfunction and can result from either a direct or secondary reaction of oxidants with a given protein. The direct reaction concerns metal-catalyzed ROS attack on amino acids, e.g. proline (Pro), arginine (Arg), lysine (Lys) and threonine (Thr). The more abundant products from this reaction on proteins are glutamic semialdehyde for Pro and aminoadipic semialdehyde for Lys [4]. These

Abbreviations: AcLDL, Acetylated low density lipoprotein; AD, Alzheimer's disease; ADH, Alcohol dehydrogenase; ADP, Adenine dinucleotide; ADPRT, ADP ribosyl-transferase; ALDH, Aldehyde dehydrogenase; Arg, Arginine; ATPase, ATP synthase; CYP2E1, P450 cytochrome 2E1; Cys, Cysteine; DHN, 1,4-dihydroxynonene; FABP, Fatty acid binding protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GSH, Glutathione; GST, Glutathione-S-transferase; HEL, N (ε)-(hexanoyl)lysine; His, Histidine; HNA, Hydroxynonenoic acid; HNE, 4-hydroxynonenal; HSP70, Heat shock protein 70; HSP90, Heat shock protein 90; LDL, Low density lipoprotein; Lys, Lysine; MS, Mass spectrometry; NOX, NADPH oxidase; PARP, Poly-ADP ribose polymerase; PD, Parkinson's disease; PDI, Protein disulfide isomerase; PKC, Protein kinase C; Pro, Proline; PUFA, Poly-unsaturated fatty acid; ROS, Reactive oxygen species; Rpt4, Proteasome regulatory particle base subunit 4; Thr, Threonine; TMP1, Tropomyosin 1; TRPV1, Transient receptor potential cation channel subfamily V member 1; UPS, Ubiquitin-proteasomal system

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carbonylated side chains increase the overall protein hydrophobicity due to unfolding, resulting in an increased risk for aggregation [4–6]. Secondary reactions generating carbonyls on proteins can also occur. For example, protein carbonylation can result from modified aldehydes (from lipid peroxidation), such as 4-hydroxynonenal (HNE), on specific amino acids as described below. This is thought to play a major role in metabolic diseases displaying increase levels of oxidative stress [7–9]. Supporting the view that HNE-modified proteins are highly associated with metabolic alterations progression Grimsrud and colleagues identified several carbonylated proteins from obese insulin-resistant C57BI/6J mice when compared to lean and insulin-sensitive ones [8]. They identified modified proteins from several different pathways such as “carbohydrate and lipid metabolism, signal transduction, antioxidant enzymes/cell stress responses, nucleic acid metabolism, protein synthesis/degradation and structural proteins”. Remarkably, they found a crucial protein for adipose tissue homeostasis, the fatty acid binding protein (FABP) to be a specific cellular target of HNE. Altogether, this strengthens the point for obesity and insulin resistance to be mediated by the increased levels of ROS and dysfunctional HNE-modified proteins. Within this review we will describe the basics on HNE-protein modifications chemistry, HNE metabolism, the fate of HNE-modified proteins and how can HNE-modified proteins be used as biomarkers for metabolic disorders.

2. How does HNE affect proteins? Chemistry of protein modification

The chemistry of HNE-formation from n-6 fatty acids in membranes or lipid stores are described in detail in several reviews [10,11]. It seems that there is more than one pathway of HNE formation is possible. Furthermore, one has to take into account, that besides HNE also other lipid peroxidation products are formed, including malondialdehyde (MDA). All aldehydic lipid peroxidation products and in addition to these also other intermediate lipid peroxidation compounds, as oxy- or peroxy-radicals, are able to react with proteins. We will focus in this review on the HNE-modified proteins, since HNE is one of the quantitatively more important lipid peroxidation products.

Although, HNE is largely formed in membranes (or other hydrophobic compartments), the partition coefficient of HNE allows a diffusion into the cytosol or the extracellular space. Nevertheless, it can be assumed that the concentration of HNE in the cell membranes is much higher, compared to the hydrophilic cellular compartments. This implies a long lasting distribution controlled reservoir for HNE in the membranes, which will be release according to a concentration gradient. Taking this into account cellular proteins might be exposed to a flux of HNE also in conditions of abated oxidant production.

It is known for quite some time that free HNE is able to react with proteins thereby changing their conformation and altering function [12].

The reaction of the HNE with a protein can take place by two principal reactions: (i) the addition of the aldehydic group to an amino group of the protein forming a Schiff´s base undergoing further rearrangements and (ii) by a Michael addition to a nucleophile by the active C=C double bond (Fig. 1). The formation of HNE-protein adducts is rather fast and in the order of seconds or minutes with exposed protein moieties [13]. Interestingly, not every protein-borne nucleophile or amino group react with the same velocity with HNE, so that Lys, histidine (His) and cysteine (Cys) are often the most frequently modified side chains. There is a strong hierarchy in the reaction as tested in the poly-amino-acids [14]. Cys revealed the highest reactivity, followed by His and Lys and Arg, with the lowest reactivity. Interestingly, regarding poly-amino-acids no significant reactivity was found using poly-glutamine, although, that does not necessarily mean that the reaction is not possible. In fact, a specific protein moiety might create a microenvironment allowing this reaction to occur. One should also take into account that protein cofactors and ligands might take part in the modulation of reactivity towards HNE.

HNE is always seen as a bi-functional aldehyde allowing, therefore, easily the formation of cross-links within or between proteins. Since under physiological conditions or under pathophysiological situations, HNE is formed as a product in a condition of oxidative stress, it is likely that many proteins are also directly damaged by oxidants. This would lead to a partial unfolding of proteins and the exposure of hydrophobic moieties at the protein surface leading to initial protein aggregates [15–18]. These can afterwards be cross-linked easily by HNE and other bi-functional reactive metabolites. It is likely that such small cross-linked protein aggregates are the basis for the age pigment lipofuscin, also called ceroid or AGE-pigment-like fluorophores by various authors [19,20].

In a cell, about 1–8% of the total formed free HNE formed is able to modify proteins as measured by the addition of free HNE to various cells in *in vitro* systems [21]. Here, one has to take into account that under real stress conditions the cellular metabolic response might be able to metabolize the amount of formed HNE quicker, especially in a densely packed tissue, so this share might eventually change in complex tissues. It is impossible to identify exclusive HNE-protein targets. Mass spectrometry-analyses (MS) revealed literally hundreds of proteins modified by HNE and there might be more; currently below the detection limit [22,23]. In addition to that, it also seems that HNE is readily crossing membranes so that in all cellular compartments protein-HNE-adducts are detectable [24]. As mentioned earlier, the binding of HNE to proteins is modulating the protein function and is clearly a dose dependent process. For a detailed review see [25]. The results on HNE-binding and affecting protein function clearly depends on the experimental or (patho)physiological conditions. As a recent study demonstrated the detection of a HNE-proteasome moiety is time dependent, demonstrating that there intramolecular re-arrangements [26]. Some protein adducts formed might be reversible especially the ones between Cys and HNE in the presence of glutathione (GSH) [27].

The fate of HNE-modified proteins will be reviewed below in this article. However, it is worth mentioning that the proteins of the proteostasis system itself are targets for HNE-modification. So, MS-analyses revealed that heat shock protein 70 (HSP70), heat shock protein 90 (HSP90) and protein disulfide isomerase (PDI) are targeted by HNE. Furthermore, also the activity of the endosomal/autophagy system might be modified by reactive aldehydes [28]. Several studies investigated the ubiquitin-proteasomal system (UPS) as a target for HNE-modification [29–34]. This includes the 20S as well as the 26S proteasomal forms. Using MS-techniques modification sites were often demonstrated and identified. Other studies reported a decline in proteasomal activity due to HNE treatment. However, such results need to be analyzed with care. Proteasomal inhibition by HNE is generally achieved only at high HNE concentrations [34]. On the other side it might well be that since HNE is a bi-functional aldehyde and able to cross-link proteins, not HNE itself but cross-linked aggregates inhibit the proteasome. The devastating effect of cross-linked protein aggregates on proteasomal function is known for several years [35–37] and was also shown for HNE-cross-linked proteins [38,39]. In our hands, HNE alone (up to 100 μM) was unable to inhibit the proteasome, but HNE-amyloid peptide aggregates were efficient inhibitors of the 20S proteasome [40].

HNE-protein adducts are physiological products of metabolism, which might be increased during diseases in tissues or plasma [41]. Therefore, HNE-protein adducts might contribute to the pathogenesis of diseases but may be used as biomarkers (reviewed in [25,42]). This will be explored in one of the upcoming chapters.

3. HNE metabolism: the amount of protein modification

The most important degrading enzymes of HNE are glutathione-S-transferases (GST), alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH). The products generated via these enzymatic

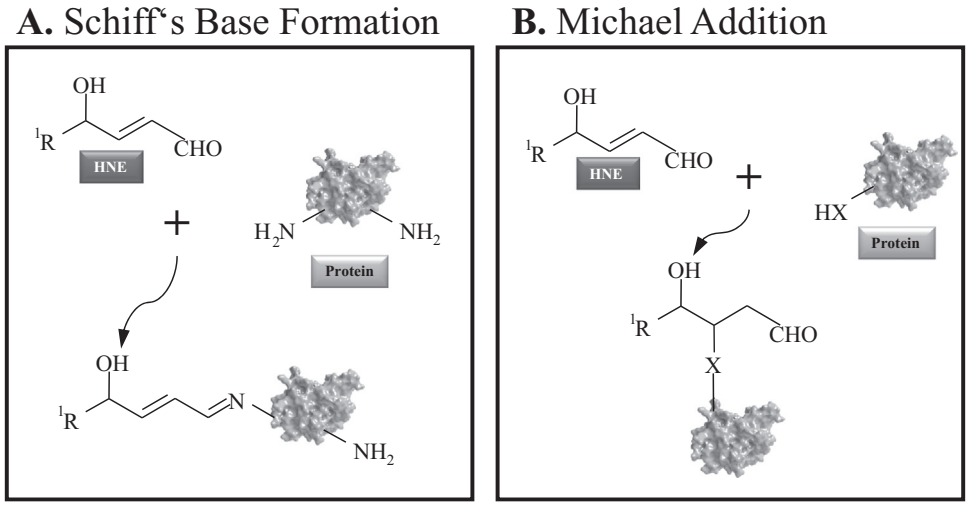

Fig. 1. HNE-modification of proteins. There are two principal ways of modification of amino acid side chains by 4-hydroxynonenal: *via* a Schiff's base formation due to the reaction of the aldehydic group of HNE with an amino group of a protein (A) or *via* a Michael addition of the HNE double bond to a protein side chain (B).

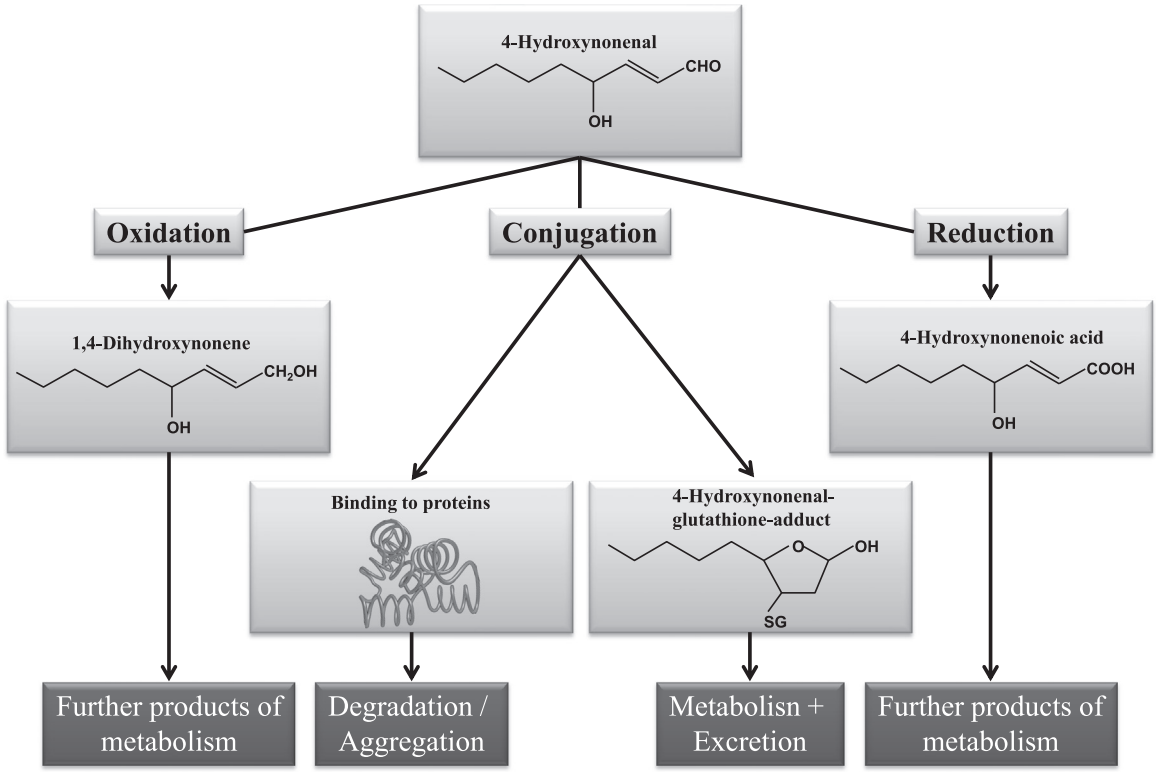

Fig. 2. HNE metabolic pathways. HNE is metabolized either by reduction, oxidation or conjugation. All pathways contribute to the HNE detoxification, with the exception of the conjugation to proteins. The formed intermediate products are either further metabolized into metabolites of the fatty acid metabolism or excreted. HNE modified proteins are either degraded or accumulate during cellular lifetime.

reactions are called primary intermediates of HNE: HNE-GSH, hydroxynonenoic acid abbreviated as HNA, and 1,4-dihydroxynonene abbreviated as DHN (Fig. 2).

The quantitative proportions with the primary intermediates of HNE in rat hepatocytes are: more than half of HNE is metabolized to the GSH-HNE conjugate, about one third is HNA, and about one tenth will be DHN. After adding HNE to rat hepatocytes after 3 min of incubation almost all HNE was degraded, and two thirds of degradation capacity was used to form the three primary HNE metabolites. One third of HNE degradation products one could find within the secondary intermediates including proteins modified by HNE. The amount of HNE-modified proteins generated in hepatocytes from exogenously added HNE was less than 10% [43].

In many experimental set-ups the concentration of HNE-protein conjugates declined again after going through a maximum after the stress exposure. That is the result of proteasomal degradation of HNE-modified proteins, comparable to other oxidatively modified proteins [18,44,45]. The pool of HNE-peptide and HNE-protein conjugates reflects an important part of damaging effects of HNE towards cellular functions. In contrast, the other pathways of HNE degradation contribute to the detoxification of HNE. Those products include products of beta- and alpha-oxidation and of the tricarboxylic acid cycle such as acetyl-CoA, citrate, aconitate, malate, fumarate, succinate, finally carbon dioxide, and water [46]. Alary and group determined and quantified GSH-HNA and GSH-DHN metabolites [47]. From those conjugates finally the mercapturic acids were formed. Very active in the

formation of the chemically stable mercapturic acids are the kidneys [13,48]. Mercapturic acids are excreted by the urine (Fig. 2).

The inhibition of different intracellular enzymes was studied by our group and others, including the Na^+K^+ ATPase, poly-ADP ribose polymerase (PARP), Complex I of the respiratory chain, protein kinase C (PKC), NADPH oxidase (NOX), and proteasome. Identified inhibition values in the literature indicate a wide range of enzyme sensitivities towards HNE (see also [18,49]). Interestingly, ADP ribosyl-transferase (ADPRT), and NOX in leukocytes are very sensitive enzymes towards HNE.

Usually inflammation is accompanied by the formation of HNE-protein-adducts this includes also atherosclerotic lesions. Many of these studies were performed by using various antibodies, however, for the detection and identification of HNE-modified proteins the high resolution MS-analyses need to be applied. Due to the low sensitivity of these methods in order to identify minor protein modifications sample enrichment procedures and new routines as neutral loss scanning should be used [11].

4. The fate of HNE-modified proteins

In the course of metabolic diseases there is an increase in HNE-modified proteins as we have described earlier. The question that then rises is what is the fate of a HNE-modified protein? One of the possible fates is degradation.

In spite of the possibility for cells to metabolize HNE when formed, a HNE-covalent modification followed by rearrangement reactions in proteins might be irreversible; therefore, in order to prevent the risk for HNE-modified protein accumulation their degradation is required. Cells employ the 20S proteasome to clear oxidized proteins, and since HNE-modified proteins also display carbonylated added groups to some of their amino acid residues such as Cys, Lys and His as described above, it is expected them to be degraded by the 20S proteasome as well (Fig. 3). However, there are isolated reports showing a role of the ubiquitination pathway for the degradation of HNE-modified proteins, as shown for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [50] without depicting whether this is targeting towards the 26S proteasome or the autosomal/lysosomal degradation pathway.

Supporting this view, the HNE-modified proteins have been shown to be degraded *in vitro* by the 20S proteasome, the known proteasomal form for degrading oxidized proteins [18,51,52]. However, this proteolytic susceptibility may vary depending on the concentration of HNE used and the stage of HNE-protein modification. In fact, data obtained from isolated histones and from Clone 9 liver cells showed that depending on the HNE concentration used, proteolytic susceptibility actually differed. For example, protein degradation was higher when mild concentrations such as 1–10 μM were used in opposite to high concentrations such as 100 μM where proteolysis was inhibited [34]. In support of this: when equine ADH was subjected to HNE in 2 fold molar excess proportion, degradation was more efficiently, around 1.5 fold faster than controls. However, when ADH was challenged with higher concentrations up to 100-fold molar, its degradation was highly impaired [53]. This diminishment in proteasomal activity might be attributed to post-translational modifications of the system itself, as has been shown that the proteasome is a target for HNE attack [29,54]. The 26S proteasome, mainly responsible for ubiquitinated protein turnover, may also have its function impaired, driven by HNE binding. A study using rats with ethanol on their diet, which presumably leads to HNE formation through P450 cytochrome 2E1 (CYP2E1) metabolism, showed from liver samples that adducts with 26S proteasome subunit proteasome regulatory particle base subunit 4 (Rpt4) were formed [32]. Decreased activity was proposed to be associated with the blockage between 19S regulator and 20S proteasome due to the adducts formed. Moreover, in agreement with these studies, a decline in trypsin-like (but not chymotrypsin-like) activity of the proteasome in HepG2 cells overexpressing CYP2E1 was related to increased levels of HNE-modified proteins compared to control [33]. Another study reported a different modification for a distinct subunit resulting in this

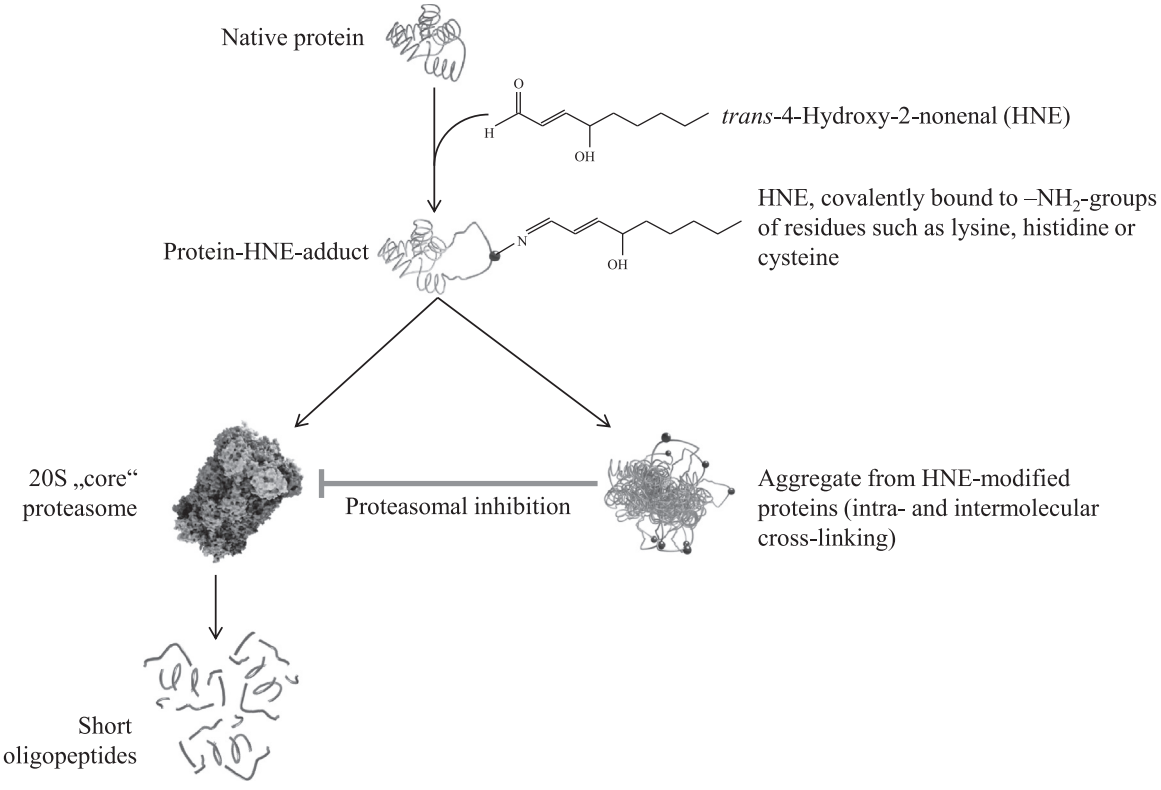

Fig. 3. The fate of HNE-modified proteins. HNE-modification of proteins might occur via a one side reaction or at two sides, due to the bifunctionality of the HNE molecule. A two side reaction might lead to intra- or intermolecular cross-links. While not cross-linked, modified proteins are readily degraded, cross-linked proteins accumulate and in turn inhibit the proteasomal system.

case, in proteasomal chymotrypsin-like activity impairment. An inhibitory effect of HNE in a specific catalytic site from the α6/C2 subunit of the 20S proteasome in liver was reported [31].

Nevertheless, there is a very limited number of studies showing different ways for HNE-modified proteins degradation. One study employing epithelial lens cells showed that when delivered into cell culture, HNE-modified crystallin was degraded at a faster rate than the unmodified crystallin and that the inhibition of the lysosomal activity stabilized HNE-modified crystallin, however, inhibition of the proteasome activity alone had only slight effects on HNE-modified crystallin stability [55], suggesting a role for lysosomal degradation or other proteases for extracellular HNE-modified proteins.

Moreover, another mechanism for degrading HNE-modified proteins was shown. A study using cochlear spiral ligament fibrocytes showed a disrupted network attributed to the HNE-modification in the proteins responsible for gap junction-mediated intercellular communication and these proteins were degraded by a calpain-degradation dependent mechanism [56].

The different types of HNE-modified degradation might be tissue or cell type dependent and further research is needed to clarify the HNE-modified protein degradation. To our knowledge there is no further mechanism described for their clearance, also no differentiation of the degradation pathway depending on the site and nature of the protein-HNE interaction is known.

Protein reaction with HNE is frequently related to the formation of cross-linked material, including various fluorophores. A relevant fluorophore has been identified as a lysine-derived dihydro-pyrrole derivative [57]. So, it seems that another fate for HNE-modified proteins might be the accumulation into cross-linked aggregates that might play a role in cellular dysfunction such as the proteasomal system inhibition. If this is the case, these HNE-driven aggregates affect the normal functioning and metabolism of cells. It is known for several years that oxidized, aggregated proteins are a poor substrate for proteasomal degradation and that they can bind to proteasome domains leading to entry clog and resulting in proteasome inhibition [58–60]. Interestingly, HNE-modified protein aggregates also seem to be detrimental to the proteasomal system, as it was shown that HNE-crossed-linked amyloid β peptide, which is responsible for the formation of senile plaques in AD, can inhibit the proteasome [40]. Several age-related neurological diseases exhibit the accumulation of protein aggregates that impair the proteasomal system. One of the consequences of decreased proteolysis is the impairment of transcription factor turnover possible triggering the apoptotic pathway [61] and the disruption in homeostasis [62]. This shows the importance of degrading on time the formed HNE-modified proteins.

Therefore, since HNE-modified proteins have been found in several metabolic diseases, their utility as biomarkers has to be considered. This will be explored in the following chapter.

5. HNE as a biomarker, HNE-modified proteins and their pathological importance

The degradation of HNE is an important antioxidative defense mechanism. It leads to the formation of non-toxic metabolites or less toxic products, which are excreted. Although, as discussed above HNE-modified proteins have a higher proteolytic susceptibility and are subject to degradation, the extracellular compartment is less endowed with proteases recognizing damaged, globular proteins and, therefore, HNE-modified proteins might be relatively stable. So, HNE-modified proteins can be used as biomarkers of oxidative stress, due to their higher biological stability, compared to free HNE or even the oxidizing radicals itself. Interestingly, HNE-modified proteins were detected in a number of diseases, e.g. in rheumatological diseases and other diseases of autoaggression [41]. A consistently growing evidence of increased HNE levels in tissues and biological fluids from a great variety of human diseases certainly suggests a pathogenic contribution by the aldehyde to their clinical expression and possible progression. It is fully recognized that HNE may take part in cell signaling involved in inflammatory reactions, which in fact represents the main driving force on the progression of the large majority of human chronic diseases [63]. Moreover, at least in terms of molecular pathology, no doubt exists about a possible causative role of the n-6 poly-unsaturated fatty acid (PUFA) peroxidation end-product HNE in cell death, inflammation, fibrosis and atypical cell proliferation [63]. Thus, while a conclusive definition of HNE role in human pathology is yet to be fulfilled; it is a matter of fact that HNE levels are increased in a number of diseases. Interestingly, HNE levels also show a tendency to increase with age as shown in a relatively small human cohort [64].

In several diseases the role of lipid peroxidation and, therefore, HNE has been demonstrated. In gastrointestinal diseases several lipid peroxidation products have been determined. So, N (ε)-(hexanoyl) lysine (HEL) and HNE-modified proteins, which are recognized biomarkers of lipid peroxidation, have been shown to in models of gastrointestinal diseases [65]. The same authors also mentioned HNE-modification of the transient receptor potential cation channel subfamily V member 1 (TRPV1) channel, HEL-modification of tropomyosin 1 (TMP1), and HEL-modification of gastrokine 1. Furthermore, the oxidative modification of LDL has been implicated in the pathogenesis of atherosclerosis. Kumano-Kuramochi et al. found that HNE-modified BSA potently inhibited the uptake of acetylated low density lipoprotein (AcLDL) [66]. Furthermore, HNE-modified LDL is playing a direct role in the pathological processes within the atherosclerotic lesion [67].

Barnham et al. and Jomova et al. described oxidative stress by including the importance of aldehyde modified proteins in neurodegenerative diseases [68,69]. Today it is widely acknowledged that changes in the redox status are involved in the pathogenesis of neurodegenerative diseases, such as Alzheimer's (AD) or Parkinson's disease (PD) [70–73].

Aldehydic products reacting with proteins might lead to the formation of immunogenic biomolecules. So, the oxidation of proteins or their lipid-peroxidation product mediated modification products might be one of autoantibody formation in chronic inflammatory diseases. Additionally, oxidatively modified LDLs also play a role in systemic lupus erythematosus (SLE) [74].

The findings by Toyoda et al., provide evidence to suspect an etiologic role of lipid peroxidation in autoimmune diseases [75]. Accumulation of modified proteins has been found by these authors in cells during aging and oxidative stress and in various pathological states, including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis. HNE-modified proteins and anti-DNA autoantibodies were demonstrated to be a serologic hallmark of SLE. Besides autoantibodies also HNE-modified immunoglobulins might be used as an indicator for oxidative stress in SLE [41]. Interestingly, in this study a correlation between the presence of HNE-modified immunoglobulin G with the acute stage of the disease was found. The potential use of HNE-modified proteins as biomarkers for some diseases is summarized in Table 1.

Table 1
HNE-modified proteins as potential biomarkers for several diseases.

Disease	References
Insulin resistant obesity	[8]
Rheumatological diseases	[41]
Neurodegenerative diseases (generally)	[68,69]
AD and PD (specifically)	[70–73]
SLE	[74]
Atherosclerosis	[76]
Gastrointestinal diseases	[65]

6. Conclusion

This review briefly summarizes the multiple effects of HNE on the cellular protein pool. It is obvious that the high reactivity of the aldehydic metabolite results in multiple effects on different proteins, changing their function and stability. High levels or formation rates of HNE might be, therefore, influencing cellular function and behavior. The formation of cross-linked HNE-protein adducts might significantly influence the cellular senescence process and, therefore, contribute to organismal aging. Nevertheless, the detection of HNE-protein adducts are opening a new variety of biomarkers significantly easier to determine and more stable compared to free HNE or even the oxidants itself.

Therefore, it is important to develop new tools and apply modern technologies in the investigation of HNE-modified proteins in order to reveal their functional impact on cell metabolism and to be used as biomarkers.

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