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Signaling by 4-hydroxy-2-nonenal: exposure protocols, target selectivity and degradation

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Abstract

4-hydroxy-2-nonenal (HNE), a major non-saturated aldehyde product of lipid peroxidation, has been extensively studied as a signaling messenger. In these studies a wide range of HNE concentrations have been used, ranging from the unstressed plasma concentration to far beyond what would be found in actual pathophysiological condition. In addition, accumulating evidence suggest that signaling protein modification by HNE is specific with only those proteins with cysteine, histidine, and lysine residues located in certain sequence or environments adducted by HNE. HNE-signaling is further regulated through the turnover of HNE-signaling protein adducts through proteolytic process that involve proteasomes, lysosomes and autophagy. This review discusses the HNE concentrations and exposure modes used in signaling studies, the selectivity of the HNE-adduction site, and the turnover of signaling protein adducts.

Keywords

HNE; redox signaling; oxidative stress; concentration; adduct; turnover

1. Introduction

4-hydroxy-2-nonenal (HNE) is a major α, β-unsaturated aldehyde derived from the decomposition of peroxidation products of omega-6 polyunsaturated fatty acids such as arachidonic acid and linoleic acid [1-5]. Besides being produced from the non-enzymatic peroxidation process, which has been well recognized and reviewed [3-6], HNE could also be generated enzymatically by cyclooxygenase-2 and lipoxygenase [7]. In addition, cells/ organisms may be exposed to HNE from food. HNE produced from dietary polyunsaturated fatty acids during food processing and storage [8, 9] could result in exposure of cells in the gestrointestinal tract and possibly enter circulation [10] (Fig.1). HNE features two functional groups, its carbonyl (–HC=O) and double bond (C2/C3, –C=C-) groups. This combination in conjugation makes HNE to react readily with bio-molecules including lipids, nucleic acids, and proteins, that can underlie oxidative damage [5]. Therefore, HNE has been widely recognized as both a marker of oxidative stress and the culprit in damage since its discovery

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in the 1980s [3, 11-13]. Later, numerous studies have clearly shown that at physiological concentration HNE can act as a potent signaling messenger and be involved in the regulation of a variety of signaling pathways [14, 15], cellular processes and functions [16], through forming Michael and/or Schiff base adducts with signaling proteins including receptors, protein kinases, phosphatases, and transcription factors [13, 17].

Most studies on HNE-induced signaling are conducted in cell models and use a wide range of HNE doses. Some doses are far beyond the pathophysiological concentration of HNE and thus might be irrelevant to what happens *in vivo*. In addition, although the adduct reaction of HNE with cysteine, histidine, and lysine residues in proteins has been a focus of many studies, the selectivity and turnover of the HNE adducts of signaling proteins have not been summarized. In this review we intend to summarize the concentration of HNE in cells/ tissues and *in vitro* cell models used for cell signaling studies, and discuss the adduction sites of HNE within signaling proteins and the turnover after adduct formation.

2. Tissue concentration of HNE

2.1. Metabolism of HNE is cell/tissue dependent

HNE in the cells is rapidly degraded through several metabolic pathways including conjugation, reduction, and oxidation [18] (Fig.1). Conjugation with GSH, catalyzed by GST alpha isoforms especially GSTA4-4 [19], is the predominant pathway of HNE metabolism [18, 20-22] and responsible for at least 50% of HNE degradation in cells [21, 23, 24]. HNE biotransformation in the other pathways leads to its oxidation to 4-hydroxy-2-nonenoic acid (HNA) by aldehyde dehydrogenase [25, 26], or its reduction to 1, 4-dihydroxy-2-nonene (DHN) by alcohol dehydrogenase [25] and aldo-keto reductase [27]. With reduction, HNE loses its ability to conjugate with proteins [28]. But even though the metabolic removal of HNE is efficient, 2-8% of the HNE in cells appears to form conjugates with proteins [18], and initiates signaling events.

HNE metabolism activities vary among tissues and cell types. Esterbauer *et al.* reported that rat liver exhibited the highest HNE metabolizing activity among liver, lung, brain, heart, kidney, and intestine. The last had less than 3% of the HNE metabolizing activity of liver, largely due to the lack of alcohol/aldehyde dehydrogenase activity in these tissues [25]. Consistently Zheng *et al.* also found that lung and brain in rat and mouse showed limited activity of HNE degradation by alcohol/aldehyde dehydrogenase compared to liver [28]. In addition, GST expression level and activity also vary with tissues [28]. Such variation in HNE metabolizing activity in different tissues means that HNE concentration may vary from tissue to tissue *in vivo*. HNE may have a relatively longer half-life in some tissues and could possibly result in higher and sustained HNE signaling in these tissues. On the other hand, a lower capacity for HNE metabolism may reflect a lower rate of lipid peroxidation and not result in a greater steady state level of free HNE. It is important to note that HNE metabolism may be altered during ontogenesis. For instance, Baradat et al. reported that compared to wild type cells, isogenic colon cells with a mutation on the adenomatous polyposis coli (APC) gene were more efficient in metabolizing HNE, due to a higher expression of HNE metabolizing enzymes [29]. Cancerous cells usually express relatively higher levels of antioxidants including GST [30] and thus potentially metabolize HNE at a

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faster rate compared to normal cells. The relatively higher HNE metabolism capacity results in a shorter HNE half-life and less HNE toxicity in cancerous cells. On the other hand it suggests that more HNE production may be required to initiate a similar signaling response in cancerous cells. Regardless, the tissue-dependent differences in HNE metabolism may result in variation of HNE levels in different cells/tissues under physiological conditions.

2.2. Tissue concentration

Free HNE remains at very low level in plasma, cells, and tissues under physiological condition. In human plasma it is in the range of 0.28-0.68 μM, similar to that in the plasma of dogs and rats [1, 3, 31]. This persistent existence of free HNE [32] under normal physiological condition may reflect a homeostatic range between its production and metabolism [33]. Plasma HNE may mainly come from tissue cells including vascular endothelial cells and hepatocytes, and blood circulating cells such as lymphocyte and erythrocytes. HNE concentration in rat hepatocytes is in the range of 2.5 μM-3.8 μM, calculated from reports that HNE in rat hepatocytes is 0.86-1.3 nmol/10^8 cells and that a typical hepatocyte volume is 3.4×10^{-9} cm^3 [34]. HNE in human blood monocytes is 3 times higher than in rat hepatocytes [3]. In other types of cells, HNE concentration is also much higher than its plasma level [5]. Since plasma HNE can reach most tissue cells, it is a fairly good indicator of the exogenous exposure level of cells throughout the body. Due to a tissue-dependent HNE metabolizing capacity as discussed in the above, HNE concentration in different tissues may vary under physiological conditions.

Under conditions of oxidative stress and diseases, HNE level is significantly increased in plasma and tissues [3, 11-13]. In most of these studies, relative level of HNE-protein adduct, instead of free HNE concentration, is usually used as HNE marker, therefore most often only the relative comparison to healthy controls was available. Nonetheless, studies have demonstrated the increase of HNE in diseases and pathologies including Alzheimer disease [35-37], cancer [38], COPD [39], and cardiovascular diseases [40], as summarized in many excellent reviews ([16, 41]).

3. HNE doses used in cell signaling study

A wide range of doses has been used in studying HNE effects on cell signaling pathways. At concentration as low as 0.01 μM HNE was able to reduce endothelial cell junctional communication [42], activate G protein mediated signaling [43], and increase phosphoinositide-specific phospholipase C (PLC) activity and neutrophil migration [44]. On the other end, HNE level as high as 4 mM was used to investigate the effect of HNE on Ca^{2+}-ATPase activity in rat liver plasma membrane [45]. The second highest concentration of HNE in the cell signaling literature was 500 μM, at which it inhibited Ca^{2+}/Mg^{2+}-ATPase activity on erythrocyte membrane [46] and Na^{+}/K^{+}-ATPase activity in rat striatal synaptosomes [47]. Analysis of literatures on HNE-mediated signaling showed that about 17% of studies used 0.1-1 μM of HNE, which is in the physiological range, and that 29% of studies used 1-10 μM of HNE, which is in the range where pathology begins. Another 38% of studies were performed with a pathological level of HNE (10-50 μM). Overall most studies (84%) have used HNE in doses from 0.1-50 μM (Table 1).

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4. Exposure mode of HNE

Exogenous addition of an agent to cultured cells to mimic the *in vivo* exposure is always a challenge. In a review article Forman discussed the pros and cons of the use in cell model systems of exogenous application of nontoxic concentration of H_2O_2, a well-recognized second messenger in redox signaling [48]. HNE shares many aspects with H_2O_2 in terms of production and metabolism in signaling studies. Both are generated *in vivo*, with a higher loci concentration, and are degraded rapidly, producing a large gradient of concentration. Therefore the challenge of HNE exposure mode in signaling studies is similar as H_2O_2 -that is to mimic the physiologically relevant intracellular concentration at the loci of target. Indeed, the physiologically relevant target would need to be close to the site of HNE generation in the cell. But, few studies have examined the effect of endogenously generated HNE.

Most HNE signaling studies are performed in cell model systems and a variety of exposure modes have been applied. In this model, cells are usually cultured in medium containing 10% or less fetal bovine serum (FBS), in some cases in FBS free medium or buffers. But in general, HNE is applied to cells through two modes; i.e., bolus one-time addition or repeated addition with intervals for several times. To reach its target signaling proteins, HNE has to escape the scavengers in serum-containing medium, the plasma membrane barrier, and the intracellular degradation system. Bolus-added HNE disappears rapidly in typical 10% FBS cell medium (80% disappears in 30 min) [49, 50], and thus HNE doses much higher than pathophysiologic level were usually used to cause effects in these studies. In other words, HNE reaching substrate-signaling molecules is obviously lower than the initial HNE concentration in the medium.

To mimic a stable HNE level for longer exposure time as is observed *in vivo*, many studies treat cells with a repeated additions of HNE [50-55]. Such an exposure mode was first used by Barrera *et al.* to investigate HNE effects on cell differentiation [56]. This group systematically measured and compared HNE concentration in cell medium after bolus or repeated addition of HNE [57]. When 10 μM HNE was added to RPMI medium with 10% FCS, 40% disappeared within 10 min; and after 30 min, HNE concentration in the medium was maintained at 4-6 μM for 1 h, indicating that HNE could be consumed by components in FCS. When 10 μM HNE was added to K562 cell suspension (10^6 cells/ml) in 10% FCS medium, it disappeared completely in 1h (undetected), with 78% having disappeared in the first 10 min (2.2 μM in medium). On the other hand, when 1 μM of HNE was added every 45 min for 12 times into cell suspension in 10% FCS medium, HNE concentration in the medium remained stable at 1 μM [57]. Similarly, Laurora *et al.* also measured the HNE concentration in medium with the repeated addition of 1μM HNE to cells in 10% FBS medium every 45 min for 10 times, and found that HNE concentration in the medium could be maintained at around 2 μM [50].

Compared with bolus exposure, the advantages of this exposure mode are obvious. First, a stable HNE level is maintained for a longer period of time, more like the *in vivo* exposure situation; secondly, potential influence on cellular response resulting from serum-free condition could be avoided; and thirdly, a kinetic response could be detected during the

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exposure period. In addition, accumulated HNE effects could be studied at lower HNE doses. However, since it takes time to reach a stable HNE level, it is inappropriate to investigate the initial targets and acute effects of HNE. In this case, bolus exposure seems more appropriate.

The biological effects of HNE are closely related to its concentration. At physiological levels, HNE is metabolized efficiently and at low intracellular concentration is maintained. Thus, its biological effects, if any, are barely observed. Under challenging conditions, where its concentration increases, HNE could act as signaling mediator and initiate various signaling cascades and regulate gene expression. At non-lethal but stressful concentrations, HNE induces processes including autophagy, senescence, and cell cycle arrest due to its pathologic modification of proteins and organelles. At lethal concentrations, HNE causes apoptosis or necrosis [58].

5. Selectivity of HNE modification on signaling proteins

HNE mediates cell signaling mainly through forming adducts with signaling protein molecules that results in change of protein activity. Cysteine, histidine, and lysine are the most active amino acids to react with HNE and proteins containing these residues could form Michael and/or Schiff base adducts with HNE [3, 5, 52, 53]. At the beginning Anti-HNE adducts Ab and protein activity assay were used to assess HNE adduct formation and effects, and many HNE targeted proteins were identified with these approaches [59, 60]. The development of mass spectrometry (MS) and proteomic-based approach in the past decade has greatly enhanced the investigation of HNE-protein reaction. With the combination with other technologies such as Click chemistry, MS analysis becomes a power tool to profile HNE targets and spot specific modification sites [54-56].

Protein oxidation, including protein glutathionylation, nitration, and other types of electrophile–protein reactions, exhibits a significant degree of selectivity, which is assumed to be due to protein structure and location [61, 62]. Studies have identified many protein substrates of HNE conjugation, and revealed that HNE-protein reactions do not occur indiscriminately, instead similar to other protein oxidation reactions, exhibit a significant selectivity at several levels.

First, only proteins with residues of cysteine, histidine, or lysine are potential targets, proteins composed of residues other than these are far less likely to form covalent adducts with HNE, as evidenced in studies with model peptides [63] and proteins [64, 65] (Table 2). The covalent modification is mainly through Michael adduction while Schiff base adduction has been less detected.

Secondly, reactivity of the three amino acids residues with HNE is different. Among them, Cys is the most preferred, and the reactivity follows the order of Cys>His>Lys [63, 65]. However, this is not absolute and in some signaling proteins, His or Lys is preferred other than Cys, such as in signaling proteins of insulin [66], ERK1/2 [67], and serine/threonine kinase liver kinas B1 (LKB1) [68]. It should be noted that controversy exists on the HNE modification sites and reactivity even in the same protein. For example, Aldini *et al.*

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investigated the reactivity of the nine residues of Cys, His, and Lys in human serum albumin [69] with HNE and found it followed an order of Cys-34 (Michael adduction) >Lys-199 (Schiff's base adduction)>His-146 (Michael adduction) [65], while Szapacs *et al.* reported that the reactivity of these residues followed the order of His-242 > His-510 > His-67 > His-367 > His-247 [70]. Both studies used similar condition (recombinant pure HSA and similar reaction ratio of HSA: HNE) and mass spectrometry analysis. It seems that the selectivity difference is related with experiment conditions such as reaction concentration and time. Uchida *et al.* reported that HNE preferentially reacted with residues of Cys and Lys in GAPDH protein at concentration of less than 0.5 mM, while it reacted with all three residues at concentration of 2 mM [64]. Ishii *et al.* on the other hand, reported that the reaction of Cys, His, and Lys in GAPDH were time dependent. Both His-164 and Cys-281 were very rapidly modified at 5 min, followed by Cys-244 at 15 min and His-327 and Lys-331 at 30 min, while the modification of Cys-149 at the catalytic center was not observed [71]. These controversies in reactivity and modification sites suggest that more studies are required to further elucidate the HNE alkylation sites of specific proteins, especially using models with similar condition as *in vivo* exposure, since most previous data were based on isolated pure protein and used HNE concentration that was not physiologically relevant. Another aspect about selectivity of HNE modification is that specific sequence motifs in proteins or secondary structure may be required for reaction of residues of Cys, His, or Lys with HNE. As observed in HSA [65, 72], Trx[73], human carboxylesterase1 [74] and other proteins that contain several residues of Cys, His, and Lys, only certain residues were able to adduct with HNE, and even these reactive residues exhibited different reactivity. The underlying mechanism of this selectivity remains largely unknown. Using computational modeling analysis, Aldini *et al.* showed that the reason why Cys374 of actin was the preferred site of HNE adduction was because of its significant accessible surface and substantial thiol acidity due to its particular microenvironment surrounding [75]. Szapacs *et al.* investigated the reactivity of HNE adduct residues and motif structure of HSA and found that the rate constants of His residues ranged over 4 orders of magnitude with the order of reactivity being His-242 > His-510 > His-67 > His-367 > His-247. The most reactive site H242 was located in a fatty acid- and drug-binding cavity of HSA. Further analysis of adduction kinetics together with HSA structure and pK(a) values of target residues suggested that location in the hydrophobic binding cavity and low predicted pK(a) of His-242 could account for its high reactivity toward HNE [70]. The relation between reactivity (selectivity) and motif sequence is further supported by a study from Doorn *et al.* [63], in which addition of a methionine to peptides significantly increased the reactivity of HNE reactive residues contained in them. Recently Yang *et al.* developed a chemoproteomics platform employing a novel, isotope-labeled Az-UV-biotin reagent and analyzed HNE alkylation sites on cysteine and histidine residues in about 400 proteins and revealed a characteristic sequence motif of CxxxK for HNE S-alkylation [76]. This powerful tool has a potential to expand the inventory of HNE modification sites of signaling proteins in complex biological samples. It is important for characterizing the interactions of HNE with redox sensitive cell signaling proteins and understanding how it may modulate their activities under either physiologic or disease conditions. With the combination of computation-based structure analysis, this technology would greatly further the understanding of the site selection of HNE covalent adduction in signaling proteins.

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Summarizing this section, it is important to understand that while the rate of adduct formation is much greater for Cys than the other two amino acids, the thiolate form of Cys (S^-) is by far a better nucleophile than the thiol (SH) form. Nonetheless Cys, and particularly its thiolate form, are in lower abundance than either His or Lys in proteins. Furthermore, even with its far greater rate constant, Cys in its thiolate form is usually less accessible to adduction than are Lys or His that would tend to be at the protein/solvent interface.

HNE concentration would also affect which amino acids are modified. Aside from the accessibility issue for the Cys thiolate, at low concentrations of HNE, its modification would be greatly favored. When the concentration of HNE is high however, Cys adduction is still the favored reaction kinetically, but the likelihood of adduction to HNE with His and Lys increase. The bottom line is that while kinetics would largely favor Cys modification, geometry and relative abundance limit the modification of Cys, and that higher concentration of HNE makes Lys and His modification increase. Thus, it is important to determine the modifications for each individual target protein at realistic concentrations of HNE.

6. Stability and turnover of HNE-adducted signaling proteins

Protein modification by HNE is often associated with conformation change and loss of normal function. The accumulation of these non-native proteins could lead to harmful effects and is associated with pathologic changes. Therefore, HNE-adducted proteins are labeled as abnormal by cell protein quality control system, and are either repaired, removed, or accumulate as aggregated proteins in cells. Although the underlying mechanism of how cells discriminate between native and oxidized form of proteins including HNE-adducts remains to be further elucidated, a growing body of evidence suggests that oxidation of amino acid residues could cause protein unfolding and exposure of hydrophobic regions, which are normally buried in native form. The exposed hydrophobic patches could serve as signals for molecular chaperones and proteolytic system, and result in the refolding or degradation of target proteins [77]. A majority of studies indicate that oxidized proteins are mainly degraded by 20S proteasome [78-80], while there are also evidence suggest that ubiquitin-dependent 26S proteasome, immunoproteasome [81, 82] and lysosome [77] are also involved in the degradation of oxidized proteins. Accumulating evidence suggests that autophagy may play a significant role in the degradation of heavily oxidized and aggregated proteins, which are poor substrate of proteasome system [83, 84]. It is generally considered that like other types of protein oxidation, HNE-modified proteins are degraded through proteasomes especially 20S proteasome. In addition, lysosome [85] and autophagy may also play a key role in the degradation and recycling of HNE-protein adducts [86, 87] (Fig.2). HNE-mediated signaling is usually transient and turned on and off in a time-dependent manner, and this is obviously related to the removal of bolus-added HNE and turnover of the signaling proteins initially adducted by HNE. Studies suggest that the appearance of HNE-signaling protein adducts is substrate dependent, some occur within minutes while others occur at as late as several hours after HNE exposure. For instance, IκB-HNE adduct level in response to uniaxial cyclic stretch reached the highest level at 2 min [88] and HNE inhibited protein tyrosine phosphatase 1B (PTP1B) activity in 5 min [89], while platelet derived

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growth factor (PDGF)-HNE adduct was detected only after 2 h of HNE exposure [81]. This time difference in the occurrence of HNE-adducts may be related to the local HNE concentration. Rinna *et al.* investigated the response of SHP1 activity to different HNE concentrations and found that at 15μM, HNE inhibited SHP1 as early as in 5 min but at 5 μM it needed 15 min to reach the same degree of SHP1 inhibition. In addition, reactivity and the location of the protein substrates may also play important roles in this time-dependent difference in adduct occurrence. To clarify the exact mechanism however, further studies are needed.

The stability or the turnover rate of HNE-signaling protein adducts also exhibit variation among target proteins. After formation, IκB-HNE adduct returned to basal level in 2 minutes [88], while HNE adducts with epithelial growth factor (EGFR) and insulin receptor substrate (IRS) remained detectable for at least 5 h [90, 91]. The presence of HNE-protein adduct however, seems to be due to accumulation other than resistance to degradation, as evidenced by both Dolinsky *et al.* [92] and Ma *et al.* [93] who observed that LKB1-HNE adduct level was higher in 1h of HNE exposure, but the total LKB1 protein was decreased. Consistently, Demozay *et al.* also found that even though the increase of HNE-IRS adducts level lasted for at least 6 h, the total IRS protein was significantly decreased [91]. However, it remains unclear whether other mechanisms such as increased secretion or inhibited translation are involved in the decrease in total proteins in the above cases. In contrast, Shearn *et al.* reported that AKT2-HNE level increased by 40 times in 2 h, and the total AKT2 protein did not change [94]. In summary, these limited data suggest that the turnover of HNE-signaling protein adducts may be substrate dependent. This point is supported by a recent study by Yang *et al.*, who investigated the turnover of 398 HNE-protein adducts using a mass spectrometry-based quantitative chemoproteomics platform and found that the adduct turnover rates varied in a site-specific manner [76]. In contrast, studies found that HNE-modified proteins usually decreased rapidly after removing HNE from medium and then remained at a stable level for longer time [85, 95]. For instance, Liu *et al.* reported that overall HNE protein adducts level decreased by 60% in 2 min of HNE exposure and then remained stable for at least 1h [87]. It remains unclear if proteasome was responsible for the rapid degradation of HNE-adducts, as it is hard to explain why the left 40% could be stable for 1 h. It is worthy to note that most HNE signaling studies are performed in cell models, which usually means short observation period and transient HNE exposure, compared to *in vivo* situation where HNE exposure persists and a possible balance between adduct formation and turnover could be reached. Further investigation of HNE-adduct turnover under these conditions would further our understanding of the mechanism of HNE-mediated signaling.

7. Conclusion and future studies

As a potent signaling mediator, HNE plays important roles in maintaining cellular homeostasis and in oxidative stress-implicated pathological changes [12, 96]. Most studies on HNE signaling in cultured cells were performed with HNE concentrations in the pathophysiologic range and whether HNE contributes to signaling effects at physiological concentration *in vivo* remains largely unknown. In addition, future studies on HNE signaling

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should consider the exposure mode; i.e., bolus-addition and repeat exposure, which is closely related to the effective HNE doses and reaction time with targets.

Selectivity of HNE adduction reaction is an important topic in the research of HNE signaling, and is relatively less studied. Most previous studies on HNE target sites and selectivity assay were conducted on recombinant protein instead of living cells, in which different HNE concentration, protein structure, and microenvironment may exist. More studies with *in vivo* models, and with mass spectrometry based technologies [76], are needed before a clear and general rule in the selectivity of HNE target can be drawn

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Highlight

- A wide range of HNE concentrations have been used in studies of HNE signaling
- Bolus and repeated addition of HNE are useful, but do not exactly mimic physiologic exposure
- HNE conjugates with and modifies signaling proteins selectively
- HNE-signaling protein adducts are subsequently degraded

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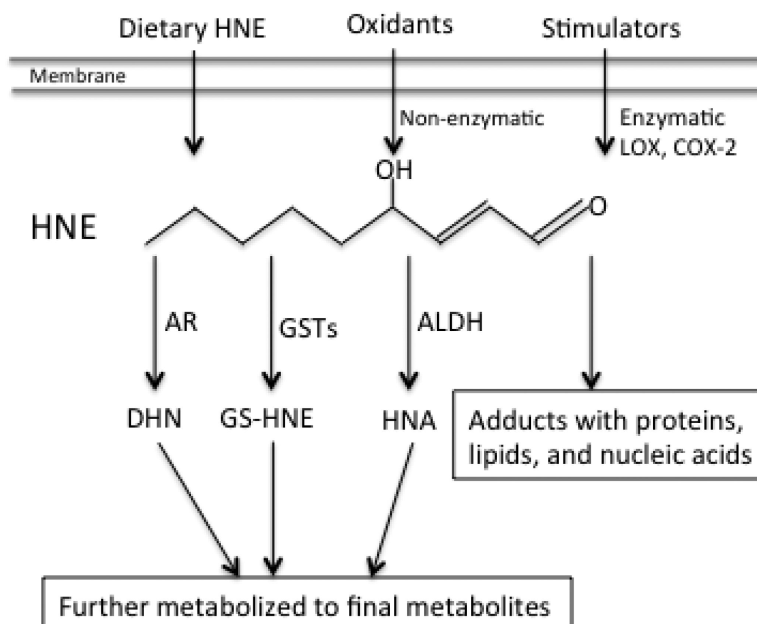

Figure 1.

HNE formation and pathway of metabolism. LOX, lipoxygenase; COX-2, cyclooxygenase-2; AR, aldo-keto reductase; GSTs, glutathione *S*-transferases; ALDH, aldehyde dehydrogenase; DHN, 1, 4-dihydroxy-2-nonene; GS-HNE, glutathione-HNE conjugate; HNE HNA, 4-hydroxy-2-nonenoic acid.

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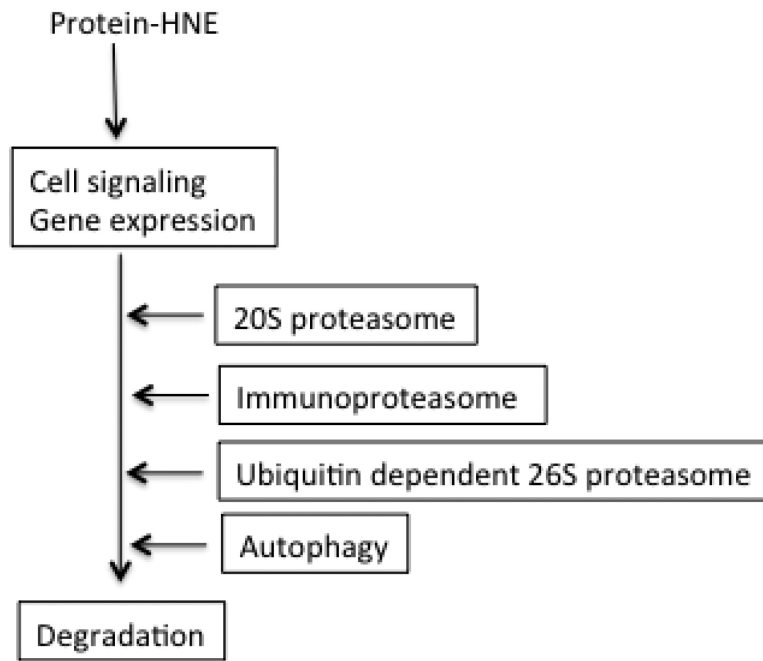

Figure 2.
Turnover of HNE-modified signaling molecules.

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Table 1

Usage of HNE concentration in signaling study

HNE concentration (μM)	Percentage of studies (%)
500	1.7
100<HNE<500	1.7
50<HNE 100	11.7
10<HNE 50	38.0
1<HNE 10	29.0
0.1 HNE 1	16.8
<0.1	1.1

Note. HNE concentration was calculated from the amount of HNE added and volume of medium or buffer and was based on 179 studies of HNE effect on signaling molecules/pathways from 1987-2015.

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Table 2

Modification of proteins by HNE[a]

Protein	Adducted Residues	Adducts Type	Reactivity	Detection method	Reference
Insulin	Two His and one Lys	Michael adduct	His>Lys	HPLC, MS, and amino acid sequencing	[66]
GAPDH	Cys, His, and Lys	Michael, Schiff base adducts, and intramolecular and intermolecular crosslink	When [HNE]<0.5 mM, preferentially with Cys and Lys; when [HNE]=2 mM react with all three residues	HPLC	[64]
β-lactoglobulin B	Cysteine, histidine, and lysine.	Mainly Michael adducts	Adducts containing from three to nine aldehyde molecules per molecule of protein	Electrospray ionization (ESI) MS	[97]
Protein kinas C (PKC)	NA	NA	NA	Anti-HNE adducts Ab	[98]
Na (+)-K(+)-ATPase	Cys and Lys	NA	NA	Anti-HNE adducts Ab	[99]
Erythrocyte membrane proteins	NA	NA	Mainly with Cys at 0-0.5 mM	Anti-HNE adducts Ab	[100]
c-Jun N-terminal kinas (JNK)	NA	NA	NA	Anti-HNE histidine Ab	[49]
FR-1	Cys298	Michael adduct	NA	ESI-MS	[95]
Tau	Lys	NA	NA	Anti-HNE Lysine Ab	[96]
Cytochrome c oxidase	NA	NA	NA	Anti HNE-histidine Ab	[101]
IκB kinas (Frikke-Schmidt, #312)	NA	NA	NA	Anti-HNE adducts Ab	[102]
Bovine cathepsin B	Cys29 and	Michael adducts	NA	Pure protein, tandem MS and Anti-HNE adducts Ab	[103]
GAPDH	His-164, Cys-244, Cys-281, His-327, and Lys-331 and revealed	Michael adducts	His-164 and Cys-281 were modified at 5 min, followed by Cys-244 at 15 min and His-327 and Lys-331 at 30 min, Cys-149 modification was not observed	Pure protein, ESI liquid chromatography-mass spectrometry (ESI-LC-MS)	[71]
Carnosine	NA	Schiff base	NA	Anti-HNE adducts Ab	[104]
Model peptides	Cys, His and Lys are modified by 4HNE;	Michael adducts	Cys>>His>Lys	MALDI-TOF-MS	[63]
Alpha 6/C2 subunit of 20s proteasome	NA	NA	NA	MALDI-TOF MS	[105]
Actin	Cys374	NA	Reactivity of Cys374 is due to a significant accessible surface and substantial thiol acidity due to the particular microenvironment	Pure protein LC-ESI-MS/MS	[75]
Protein disulfide isomerase (PDI)	Cys	NA	NA	MS	[102]
Carnosine	Cys	NA	NA	ESI-MS	[106]
Epithelial growth factor receptor (EGFR)	NA	NA	NA	Anti-HNE adducts Ab	[107]
Human serum albumin [65]	His-67, His-146, His-242, His-288, His-510, Lys-195,	8 Michael Adducts (MA), 3 Schiff Base (SB)	Cys-34 (MA)>Lys-199 (SB)>His-146 (MA)	LC-ESI-MS/MS	[65]

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Protein	Adducted Residues	Adducts Type	Reactivity	Detection method	Reference
	Lys-199, Lys-525 and Cys-34				
Thioredoxin reductase	Cys-496	NA	NA	MS	[108]
Enolase 3b, aldolase and triosephosphate isomerase 1, creatine kinase, carbonic anyhdrase III, aconitase 2, dihydrolipoamide dehydrogenase, and electron transfer flavoprotein-beta	NA	NA	NA	MS	[106]
Type II collagen and MMP-13	NA			Anti-HNE adducts Ab	[109]
HSA	10 His and Lys residues	Michael Adducts	H242 > H510 > H67 > H367 > H247	LC-MS-MS	[70]
α-synuclein	His-50	NA	NA	LC-MS/MS	[110]
ADP/ATP translocase 1	Cys-256	Michael adducts	NA	MALDI-MS/MS	[111]
Extracellular signal-regulated kinas ½ (ERK1/2)	His-178	NA	NA	LC-MS/MS	[67]
Tubulin	Cys-347, Cys-376, and Cys-303	Tubulin cross-links are Lys-dependent	NA	LC-MS/MS	[112]
HSA	Cys-34	NA	NA	LC-ESI-MS/MS	[113]
HSA	Cys-34 and Lys-199	Cys-34 (MS) and Lys-199 (SB)	NA	LC-ESI-MS/MS	[72]
Akt	NA	NA	NA	Anti-HNE adducts Ab	[114]
HSP70 and HSP90	NA	NA	NA	Click chemistry and proteomics	[115]
Liver kinas B1 (LKB1)	NA	NA	NA	Anti-HNE adducts Ab	[92]
Toll like receptor 4 (TLR4)	Cys	NA	NA	LC-MS/MS	[116]
Trx	Cys-73 and Cys-32	NA	Cys-73 > Cys-32	NMR	[73]
Type II collagen	NA	NA	NA	Anti-HNE adducts Ab	[117]
Glutamate cysteine ligase: catalytic (GCLC) and modifier subunit (GCLM)	Cys-553 on GCLC and Cys-35 on GCLM	NA	NA	Pure protein, MALDI-TOF	[118]
Human carboxylesterase1	Lys-105 and Cys-389		Only Lys-105 adducted	MS	[74]
SIRT3 deacetylase	Cys-280	NA	NA	MS/MS	[119]
AKT2	His-196, His-267, and Cys-311 of rat Akt2	Michael Adducts	NA	Anti-HNE adducts Ab and MALDI-TOF	[94]
Phosphatase and tensin homolog (PTEN)	NA	Single Michael adduct	NA	Anti-HNE adducts Ab and MALDI-TOF/TOF	[120]
EGFR	NA	NA	NA	Anti-HNE adducts Ab	[121]
LKB1	Lys-97	NA	NA	Anti-HNE adducts Ab	[68]
IκBα	NA	NA	NA	Anti-HNE adducts Ab	[122]
PKC	NA	NA	NA	Anti-HNE adducts Ab	[123]
Liver fatty acid-binding protein (L-FABP)	Lys-57 and Cys-69 on apo and Lys-6, Lys-31, His-43, Lys-46, Lys-57 and Cys-69) on holo protein	NA	NA	Pure protein, MALDI-TOF/TOF MS	[124]

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Protein	Adducted Residues	Adducts Type	Reactivity	Detection method	Reference
Peptidyl-prolyl cis/trans-isomerase A1 (Pin1)	His-157 and Cys-113	Michael adducts	Cys-113 is the primary	MALDI-TOF/TOF MS	[125]
Mitochondrial aconitase (ACO2)	Cys	Michael adducts	The most reactive sites were Cys-358, Cys-421, Cys-424, Cys-99 and Cys-565	MS	[126]
Angiotensin II (Ang II)	NA	NA	NA	Anti-HNE adducts Ab	[127]
GRP78	Lys and His	NA	Marked propensity for Lys and His adduction within the ATPase domain	MS	[128]
Lactate dehydrogenase (LDH)	His-68, Cys-164, Cys-186, and Cys-294	Michael adducts	NA	Purified protein, MS	[129]
5′ AMP protein kinase (AMPK)	Cys-130, Cys-174, Cys-227, and Cys-304 on AMPKα and Cys-225 on AMPKβ	Michael adducts	NA	Pure protein, MS	[130]
Protein kinase A	Cys-199	NA	NA	MS	[131]
Src	Cys-248	NA	NA	LC-MS/MS	[132]
398 proteins	386 Cys sites and 12 His sites	Michael adducts	NA	MS	[76]
Cyclin-dependent kinas 2 (CDK2)	NA	NA	NA	MS	[133]
Apoptosis inducing factor (AIFm2)	His-174	NA	NA	MS	[134]

Note. NA, not available; MS, mass spectrometry.