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Reactive oxygen species and α,β-unsaturated aldehydes as second messengers in signal transduction

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Abstract

Signaling by H_2O_2, α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (HNE) and related chemical species, is thought to differ from signaling by other second messengers because the oxidants and other electrophiles can readily undergo nonenzymatic reactions and are therefore classified as "reactive." This brief review will describe how and when the chemistry of signaling is similar or differs from classic second messengers, such as cyclic AMP, or posttranslational signaling, such as farnesylation or ubiquitination. The chemistry of cysteine provides a common factor that underlies signaling by H_2O_2 and HNE. Nonetheless, as H_2O_2 and HNE are rapidly metabolized *in vivo*, spatial considerations are extremely important in their actions. Therefore, the locations of sources of H_2O_2 and α,β-unsaturated aldehydes, the NADPH oxidases, mitochondria, membrane lipids, and redox cycling toxicants, as well as their targets, are key factors. The activation of the JNK pathway by HNE and endogenously generated H_2O_2 illustrates these principles.

Keywords

hydrogen peroxide; superoxide; 4-hydroxynonenal; thiol; thiolate; protein tyrosine phosphatase; glutathionylation; thioredoxin

Introduction

This review focuses on the issue of when and how reactive oxygen species, particularly superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), and reactive aldehydes, particularly 4-hydroxy-2-nonenal (HNE) can be classified as second messengers. Second messengers, such as cyclic AMP, have several characteristics. These are that the activation or inactivation of regulated enzymatic processes or channels can transiently alter their steady state concentrations and that they have specific targets. The discussion that follows suggests that H_2O_2 can be truly considered a second messenger when it is generated in response to physiological stimuli. In contrast, $O_2^{\bullet -}$ more likely acts as a precursor of H_2O_2 than an

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actual second messenger and HNE acts on signaling pathways by posttranslation modification rather than as a classic second messenger.

Reactive oxygen species as signaling molecules

Physiologically generated $O_2^{\bullet-}$

Superoxide is generated in a regulated process by the NADPH oxidases that have been described in almost all cell types.[1–3] The activities of the NADPH oxidases are themselves regulated by either assembly of cytosolic and membrane components and/or through transcription of their components. Catalysis by five of the seven mammalian NADPH oxidases involves an obligate one-electron reduction of oxygen that generates $O_2^{\bullet-}$. Although it has been known for over 35 years that mitochondria generate $O_2^{\bullet-}$,[4,5] evidence suggesting a link to signal transduction has only emerged in the last several years.[6–8] The generation of $O_2^{\bullet-}$ in mitochondria is dependent upon the nonenzymatic oxidation of ubisemiquinone. Although the expression of uncoupling proteins,[9] the metabolic state,[10] and nitric oxide binding to cytochrome oxidase can alter $O_2^{\bullet-}$ production,[6] the generation of $O_2^{\bullet-}$ by mitochondria is clearly not typical for a second messenger. Superoxide generated outside the cell can be shown to be involved in signaling by entering the cell through an anion channel[11]; however, upon entry into the cytosol it is likely to be dismuted to H_2O_2 and O_2 by superoxide dismutase. Indeed, regardless of its source, whether $O_2^{\bullet-}$ directly acts as a second messenger is questionable because specific targets have not been verified and it would have to act within a very short distance from its site of generation due to the high concentration and extraordinarily high catalytic rates ($>10^9\ M^{-1}\ s^{-1}$) of the superoxide dismutases in the cytosol and mitochondria.

Physiologically generated $H_2\ O_2$

In contrast to $O_2^{\bullet-}$, H_2O_2 clearly can act as a second messenger and $O_2^{\bullet-}$ is the immediate precursor of most of the H_2O_2 produced by cells. In mitochondria, ubisemiquinone oxidation appears to be an endergonic reaction that is pulled forward by its coupling to the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 catalyzed by mitochondrial superoxide dismutase (SOD) as was demonstrated by the acceleration of substrate oxidation by the addition of SOD to submitochondrial particles and acceleration of H_2O_2 production by overexpression of mitochondrial SOD.[5,12] Additional sources of H_2O_2 are two NADPH oxidases that are called Duox 1 and Duox 2 that catalyze two-electron reduction of oxygen.[3] In addition, a number of flavoprotein enzymes involved in metabolism generate H_2O_2 directly.[13] As with $O_2^{\bullet-}$, the physiological targets for H_2O_2 must be close to the site of its generation due to abundant presence and high catalytic rates of the peroxiredoxin (Prdx) and glutathione peroxidase (GPx) families of enzymes, in particular, that can remove them. The spatial locations for production and elimination of $O_2^{\bullet-}$ and H_2O_2 are illustrated in Figure 1. The specificity of H_2O_2 reactions for signaling, which is primarily with specific protein cysteine moieties, will be described in more detail later.

Generation of $O_2^{\bullet-}$ and H_2O_2 from xenobiotic compounds

Another source of $O_2^{\bullet-}$ is the redox cycling of toxic substances such as paraquat and quinones.[14] Various NAD(P)H-dependent oxidoreductases in cells are able to reduce these

xenobiotic compounds to their one electron reduced free radical state that is then reoxidized by transferring its extra electron to O_2 producing $O_2^{\bullet -}$.[15] Although generation of $O_2^{\bullet -}$ and H_2O_2 from such redox cycling is certainly not physiological, cells can respond in a manner that mimics physiological generation including activation of signaling pathways. An example is the induction of GSH synthesis by quinones.[16,17] On the other hand, because nonphysiological production of $O_2^{\bullet -}$ can occur in locations removed from the targets of physiologically generated $O_2^{\bullet -}$, the potential for aberrant effects on cell function rather than physiological mimicry is likely, especially when the generation from redox cycling toxicants can be much greater in quantity and duration than occurs from physiologically regulated processes. The use of exogenous H_2O_2 in studying redox signaling is subject to the same caution regarding physiological relevance.[18]

The α,β-unsaturated aldehydes as signaling molecules

Among the many products resulting from the nonenzymatic oxidation of polyunsaturated fatty acids are the α,β-unsaturated aldehydes. Esterbauer described these compounds and noted that one of the more abundant compounds, HNE, had multiple physiological effects, including involvement in cardiovascular disease.[19,20] HNE can bind to proteins through its two different functional groups (Fig. 2). The electrophilic double bond reacts with nucleophiles, the amino group of lysine, and the imidazole of histidine and most readily with the thiol group of cysteine.[21] If the cysteine is close to positively charged amino acid functional moieties, it can be dissociated to a thiolate (S^-) that is a much better nucleophile than the thiol and therefore can more readily and rapidly react with HNE by Michael addition. The Michael addition products with amines and imidazole are easily reversed whereas the reversibility of the reaction forming the sulfur adduct is less likely. Nonetheless, the unstable adduct to an amine can go on to form a stable pyrrole.[22] The other major reaction of HNE is the readily reversible formation of Schiff bases by reaction of the aldehyde moiety with an amine moiety of a protein or other cellular component.

Although HNE production is usually associated with pathology, it appears that even in the absence of any stress, healthy individuals have a significant amount of HNE in their plasma (estimated as 300–700 nM).[23,24] Much of this is likely to be in the form of Schiff bases that serve as a pool from which it can be easily released when free HNE declines. During exposure to redox cycling toxicants, inflammation or other pathologies that involve the production of reactive oxygen species, lipid peroxidation resulting in HNE production increases, and the local concentration of HNE in tissues can increase to 10 μM or more.[25] Because of its low aqueous solubility however, most HNE would partition into membranes and therefore be at much greater concentration near the membrane than measured in total tissue homogenate.

As HNE is a product of nonregulated lipid peroxidation and its adduction to proteins is nonenzymatic, it is clearly not a classical second messenger. There is however, regulation of the three major pathways through which HNE is eliminated (Fig. 3). Aldose reductase, which catalyzes the reduction of HNE to non-2-ene-1,4-diol, aldehyde dehydrogenase, which catalyzes the oxidation of HNE to 4-hydroxy-2-nonenoic acid, and glutathione S-transferase isoforms that conjugate HNE to GSH, can be all be induced by HNE but not

necessarily in all cell types.[26–29] Although the relative importance of the three pathways also varies with cell type, the conjugation of HNE with glutathione (GSH) appears to be of major importance in most tissues. Interestingly, the first enzyme in *de novo* synthesis of GSH, glutamate cysteine ligase (also called γ-glutamylcysteine synthetase) is also induced by HNE leading to an increase in GSH content.[30] Indeed, the induction of HNE metabolizing enzymes and GSH synthesis leads to the ability of cells to adapt to formerly lethal exposure to HNE when exposed to HNE at nonlethal micromolar concentrations similar to what is observed in inflammation or even repeated doses of HNE to mimic maintenance of normal plasma concentrations.[31] The induction of the HNE metabolizing enzymes indicates that an increase in HNE concentration can be sensed by cells in a manner leading to the increased transcription of genes.

Signaling targets for H_2O_2

Despite its designation as a reactive species, H_2O_2 reacts rapidly with few biological molecules. Hydrogen peroxide can react rapidly with transition metals in their reduced state, such as iron II in the Fenton chemistry producing the very reactive hydroxyl radical ($^{\bullet}OH$).[32] Fortunately, iron in cells is usually bound to proteins in a state that is not available for that reaction. Iron-containing enzymes such as myeloperoxidase use H_2O_2 as a substrate to produce hypochlorous acid that can stimulate signaling pathways[33]; however, HOCl is a more reactive species than H_2O_2 and therefore more likely to be involved in pathological processes such as the damage incurred in inflammation than in signaling. The iron-containing lipoxygenases and cyclooxygenase are intimately involved in signaling in that they catalyze the first steps in the production of the leukotrienes and prostaglandins, respectively. Cyclooxygenase and lipoxygenases do not use H_2O_2 as a substrate but can be activated by low concentrations of H_2O_2 and other hydroperoxides whereas higher concentrations inhibit these enzymes.[34–37]

In terms of a role for H_2O_2 as a second messenger, the reversible oxidation of protein cysteines has become the main focus of attention. Thiols do not react rapidly with H_2O_2 but, when ionized to a thiolate the rate of reaction increases markedly.[38] The reaction of a thiolate with H_2O_2 produces a sulfenate

$$RS^- + H_2O_2 \rightarrow RSO^- + H_2O \quad (1)$$

where R is an alkyl. How rapidly this reaction occurs depends upon the surrounding environment. The pKa of cysteine is 8.3. For a cysteine in a protein to become a thiolate requires that positively charged moieties be close to the cysteine. But, dissociation to a thiolate does not alone produce a very reactive species as the rate constant for many thiolates, such as in the active site of protein tyrosine phosphatase 1B (PTP1B) is between 9 and 43 M^{-1} s^{-1},[39–41] whereas the rate constant for reaction (1) in the active site of a peroxiredoxin is approximately 10^7 M^{-1} s^{-1}.[42–45] Therefore, if the reaction of a protein thiolate with H_2O_2 were the mechanism through which H_2O_2 acts as a second messenger, the surrounding microenvironment of other functional groups would have to assist in the reaction as it does in the peroxiredoxins. Alternatively, the concentration of H_2O_2 would

have to be very high, which would only be possible if the source of H_2O_2 was very close to the target protein.

Reaction (1) is not readily reversed. Yet, the oxidation of several signaling proteins (e.g., PTP1B, PTEN) by H_2O_2 involves reversible oxidation of a critical thiol.[41,46] There are two forms of oxidized cysteine that can be formed during signaling that are readily reduced back to the thiolate. These are an intramolecular disulfide, such as is observed in thioredoxin, and a protein-glutathione disulfide (also called a mixed disulfide), which is observed in the inactive form of PTP1B.

Formation of protein-glutathione disulfides

Two mechanisms have been demonstrated for the formation of a protein-glutathione disulfide. The first involves reaction of the sulfenate formed in reaction (1) with GSH

$$\mathrm{RSO^- + GSH \rightarrow RSSG + OH^-} \quad (2)$$

This reaction is followed by the reduction of the thiolate by a second molecule of GSH

$$\mathrm{RSSG + GS^- \leftrightarrows RS^- + GSSG} \quad (3)$$

producing glutathione disulfide (GSSG). Note that reaction (3) is reversible.

The alternative mechanism through which H_2O_2 can be used to produce a mixed disulfide involves the formation of GSSG by the action of a glutathione peroxidase (GPx) or Prdx6

$$\mathrm{H_2O_2 + GSH \xrightarrow{GP_x \text{ or } Prdx6} 2H_2O + GSSG} \quad (4)$$

followed by reaction (3) going in the right to left direction. The formation of mixed disulfides is an important intermediate in protein folding that is catalyzed by protein disulfide isomerases, such as those in the cysternae of the endoplasmic reticulum and glutaredoxin.[47,48] Without an enzyme, the rate constants for disulfide exchange in either direction of reaction (3) are very slow. Formation of mixed disulfides is also a characteristic of a major oxidative stress, which is the one situation in which GSSG is significantly greater than a few percent of the total glutathione pool in the cytosol.[49,50] This is largely because the rate of cellular reduction of GSSG catalyzed by glutathione reductase is generally far greater than the rate of GSH oxidation.[51]

Both direct oxidation and reaction with GSSG of the active site cysteine have been demonstrated *in vitro* to be possible for the formation of the mixed disulfide of PTP1B.[41,52–54] In one study, a very high concentration of GSSG added to the enzyme produced the PTP1B–glutathione mixed disulfide.[52] In other studies, the sulfenate intermediate from reaction PTP1B with H_2O_2 was implied from the formation of a metastable sulfenylamide that likely formed from the sulfenate reacting with a protein backbone amide nitrogen.[53,54] Although both of these mechanisms can be demonstrated *in vitro*, the kinetics of either reaction do not match the appearance of the PTP1B glutathione

disulfide in intact cells stimulated to produce H_2O_2.[18] Thus, the mechanism for PTP1B glutathionylation remains unresolved. Similarly, the formation of a PTEN-glutathione disulfide was also demonstrated to occur in cells stimulated to produce H_2O_2 through an unknown mechanism.[55]

Formation of intramolecular disulfides

Some proteins, notably thioredoxins, can readily form disulfides from the two critical cysteines in their active site. Thioredoxin (Trx) contains other cysteines that can be oxidized during oxidative stress; however, the cysteine that can react most readily with H_2O_2 is in the thiolate form.[56] It then reacts with the second active site cysteine to form the disulfide. The enzyme-catalyzed rate of reaction to form the disulfide is markedly accelerated compared to the nonenzymatic rate by mammalian peroxiredoxins 1–5:

$$2\,\mathrm{Trx}-(\mathrm{SH})_2+\mathrm{H_2O_2} \xrightarrow{\mathrm{Prdx\ 1-5}} \mathrm{Trx}-\mathrm{S_2}+2\,\mathrm{H_2O} \quad (5)$$

where Trx-(SH)2 is reduced thioredoxin and Trx-(S)2 is thioredoxin in the disulfide form.

In the enzyme catalyzed reaction however, it is a cysteine of the Prdx rather than the Trx that reacts with the H_2O_2 forming a sulfenate intermediate. The Trx-$(SH)_2$ reduces the sulfenate producing an intermediate protein-Trx disulfide that is resolved to the Prdx-S^- and Trx-S_2. The formation of an intramolecular disulfide has also been proposed for signaling proteins, such as the low molecular weight protein tyrosine phosphatase (LMW-PTP).[57,58] One question is whether this results from direct oxidaion by H_2O_2 or requires a catalyst for direct oxidation by H_2O_2 or occurs through disulfide exchange with the target acting in parallel to Trx in the peroxiredoxin reaction.

Trx-(S)2 can be reduced in reaction

$$\mathrm{Trx}-\mathrm{S_2}+\mathrm{NADPH}+\mathrm{H^+} \xrightarrow{\mathrm{Thioredoxin\ reductase}} 2\,\mathrm{Trx}-(\mathrm{SH})_2+\mathrm{NADP^+} \quad (6)$$

Although the rate of GSSG reduction is much greater than the rate of GSH oxidation, the relative rate of Trx reduction to oxidation is not as high. The bond forming the intramolecular protein disulfides in Trx or LMW-PTP are far more stable than that of the protein-glutathione disulfide or even GSSG. Therefore, as a result of the requirement for catalysis and the difference in relative stability of the disulfides, the thioredoxin and glutathione systems are not at equilibrium with each other and can have different redox poise even in the same compartment.[59,60] Nonetheless, GSH can restore the activity of the inactive LMW-PTP, suggesting an exchange reaction catalyzed by glutaredoxin:

$$\mathrm{Protein}-\mathrm{S_2}+2\mathrm{GSH} \overset{\mathrm{Glutaredoxin}}{\rightleftarrows} \mathrm{Protein}-(\mathrm{SH})_2+\mathrm{GSSG} \quad (7)$$

that could be driven forward by the very high GSH/GSSG ratio that is usual in cells.

Thiol-disulfide exchange reactions that do not use GSH are common in the cysternae of the endoplasmic reticulum and in mitochondria where the protein disulfide isomerases, which

have thioredoxin-like active sites, are responsible for catalyzing protein folding.[61–63] In those reactions, thiols in the protein are exchanged with intramolecular disulfides until the most stable conformation of the protein is obtained.

H_2O_2-dependent activation of the ASK1/JNK pathway

The activation of ASK1, which is a protein kinase kinase kinase that is upstream of both JNK and $p38^{MAPK}$, is regulated by the binding of one of two related proteins that inhibit the activation of ASK1.[64,65] Ask1 is activated when the bound Trx or glutaredoxin becomes oxidized allowing dimerization of ASK1 and autophosphorylation.

We had observed that c-Jun phosphorylation in alveolar macrophages stimulated with exogenous ADP was dependent upon stimulation of H_2O_2 production from the respiratory burst.[66] Going upstream from c-Jun phosphorylation, JNK, MKK4, and ASK1 were also shown to be phosphorylated and Trx to transiently dissociate from ASK1 in an H_2O_2 dependent manner following ADP stimulation. Thus, activation of the pathway from Trx dissociation to the phosphorylation of c-Jun was shown to be H_2O_2 dependent. The oxidation of Trx necessary for this to occur was too small a percentage of the total Trx to measure, suggesting that the oxidation of the Trx bound to ASK1 was likely a local event catalyzed by a Prdx using the H_2O_2 produced by the respiratory burst.

Signaling targets for HNE

As described earlier, HNE reacts nonenzymatically with amines, imidazole, and cysteine. Although the abundance of cysteine is very small compared with nitrogenous targets, its reaction rate, particularly as the much stronger nucleophilic thiolate, makes it more likely that a cysteine thiolate will be involved in signaling at concentrations of HNE that are present in the absence of an imposed oxidative stress or under mild oxidative stress. Indeed, the low level of HNE found in plasma from normal unstressed humans contributes to signaling for the “basal” expression of glutamate cysteine ligase (GCL), which catalyzes the first step in *de novo* synthesis of GSH.[31] GCL has both a catalytic subunit and modulatory subunit. An increase in expression of both genes in human bronchial epithelial cells by HNE depends upon the activation of both Nrf2 and JNK pathways.[31,67]

HNE is produced from lipid peroxidation and is lipophilic. Thus, HNE concentration would retain a steep gradient from it site of its production in a membrane (or from the plasma membrane if introduced to cells from outside) into the cytosol or inner compartments of organelles.[68] Therefore, most targets for HNE modification at physiological concentrations are proteins with reactive cysteines that are close to membranes. Location of targets is an important factor that is often overlooked in investigations of the effects of HNE on cells so that studies showing the addition of HNE to proteins include those that would not actually be modified during physiologically relevant oxidative stress. Yet, investigation of signaling by HNE requires a compromise to modify enough of the physiologically relevant target proteins to allow detection. Using concentrations that approach those found in inflammation or other pathologies can allow the identification of potential signaling targets with the caveat that more proteins will be modified and the percentage of a protein that is modified will increase. Thus, other approaches, such as silencing with siRNA while using a nontoxic

concentration of HNE, would be needed to verify a physiologically significant signaling role.

This compromise has been illustrated with Keap1, the protein that prevents activation of Nrf2 activation by facilitating its degradation. Only a small percentage of Keap 1 needs to be modified for Nrf2 activation to be detectible. Thus, activation of Nrf2 has been demonstrated with 0.5 μM HNE using siRNA for Nrf2[31]; however, at that concentration, not enough of the Keap1 is modified to allow detection of the HNE adduct by mass spectrometry. Using 20 μM HNE, which is lethal to some cell lines, does allow sufficient modification of Keap1 by HNE for determination of the site of HNE binding.[69]

Activation of Nrf2 is required for induction of both GCL genes by HNE in human bronchial epithelial cells as was demonstrated using RNA silencing; however, that is not sufficient for GCL expression. Both genes also have TRE (AP-1 binding) *cis* elements that are critical in HNE induction.[67] The activation of the TRE requires binding of phosphorylated c-Jun and JNK is responsible for that phosphorylation. HNE can activate all three major mitogen-activated protein kinase pathways in a rat lung epithelial cell line[70]; however, the actual site of its action is not known for ERK or $p38^{MAPK}$. It was reported over a decade ago that HNE could bind to a histidine on JNK and cause its translocation to the nucleus and activation in stellate cells[71]; however, this may have occurred in those cells because of their very low capacity for elimination of HNE.

In contrast, activation of JNK by HNE in human bronchial epithelial cells appears to be primarily through the inactivation of a protein tyrosine phosphatase, SHP-1.[72] The pathway from SHP-1 to JNK is not entirely known; however, both inactivation of SHP-1 by HNE or silencing of SHP-1 with siRNA causes activation of JNK and its upstream protein kinase, MKK4. The modification of SHP-1 by HNE is presumed to be at its active site critical cysteine, which is consistent with inhibition of its catalytic activity. Nonetheless, following inactivation by HNE, SHP-1 protein is degraded. Therefore, HNE modification of SHP-1 is irreversible and the signaling is analogous to ubiquination rather than more classic second messenger signaling. Furthermore, most, if not all, responses to HNE other than its own metabolism appear to occur through nonenzymatic reactions, which are markedly different from both second messenger and other post-translational modifications, such as ubiquination and farnesylation that are enzyme catalyzed.

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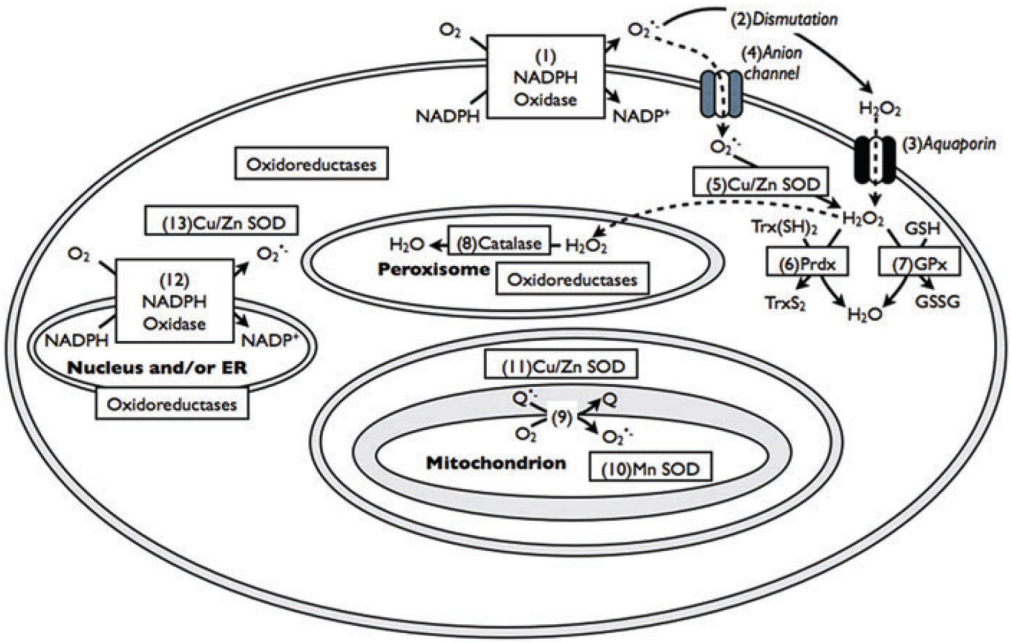

Figure 1.

Locations of superoxide and hydrogen peroxide production and degradation relevant to signaling. (1) Superoxide produced on the outside of the cell by NADPH oxidase in the plasma membrane, (2) dismutes to H_2O_2, (3) that can enter the cytosol through an aquaporin.[73] (4) Superoxide can enter cells through an anion channel where (5) it will be dismuted to H_2O_2 and O_2.[74] In the cytosol, (6) Prdx and (7) Gpx will rapidly eliminate H_2O_2. (8) Catalase present in the peroxisomes will eliminate H_2O_2 that either enters. (9) Superoxide is generated in the mitochondria from ubiquisemiquinone oxidation in a reaction that is pulled forward by (10) Mn superoxide dismutase to generate H_2O_2. If $O_2^{\bullet-}$ were produced on the outside of the inner membrane, (11) Cu/Zn SOD present there would dismute it to H_2O_2 and O_2. (12) NADPH oxidase 4 present on the nuclear and/or endoplasmic reticulum generates $O_2^{\bullet-}$ that would also be dismuted. Oxidoreductases in other compartments are potential sources of $O_2^{\bullet-}$ and H_2O_2 but are not clearly implicated in physiological signaling.

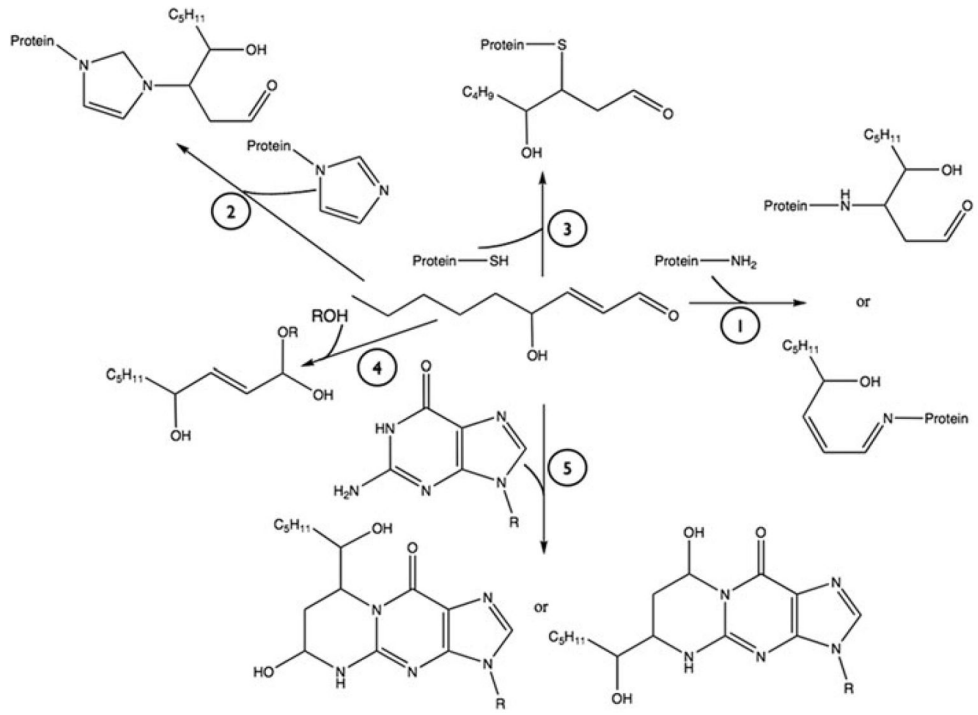

Figure 2.

Nonenzymatic reactions of 4-hydroxynonenal. HNE can react with an amine (1) to form either a Schiff base (bottom) or a Michael addition product (top). Michael addition products can form with (2) the imidazole moiety of histidine or (3) the thiol moiety of cysteine. (4) A hemiacetal can form with an alcohol. (5) Tetrahydropyrimidoguanine products can form from the reaction of guanine with both the α,β-unsaturated bond and aldehyde functional group.

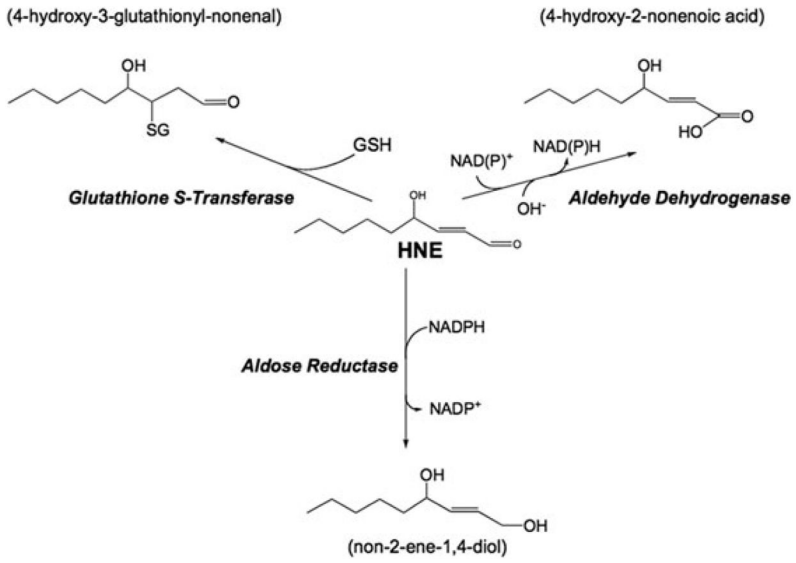

Figure 3.

Enzymatic metabolism of 4-hydroxynonenal. HNE can be reduced by aldose reductase, oxidized by aldehyde dehydrogenase, and conjugated to GSH by a glutathione S-transferase.