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Inhibition of Thioredoxin and Thioredoxin Reductase by 4-Hydroxy-2-nonenal in Vitro and in Vivo

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Abstract: Lipid peroxidation is a cellular process that takes place under physiological conditions and particularly after oxidative stress. 4-Hydroxy-2-nonenal (HNE), a major end product of lipid peroxidation, is known to exert a multitude of biological effects and has high reactivity to various cellular components, including DNA and protein. The thioredoxin system, composed of the selenoenzyme thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH, plays a key role in redox regulation and is involved in many signaling pathways. The selenocysteine (Sec) and cysteine (Cys) residues (Cys-496/Sec-497) in the active site of TrxR and a pair of Cys residues (Cys-32/Cys-35) in Trx are sensitive to various alkylating reagents. Herein, we report a mechanistic study on the inhibition of rat TrxR by HNE. The inhibition occurs with TrxR only in its reduced form and persists after removal of HNE. Inhibition of TrxR by HNE added to cultured HeLa cells is also observed. In addition, HNE inactivates reduced *Escherichia coli* Trx irreversibly. We proved that the redox residues (Cys-496/Sec-497 in TrxR and Cys-32/Cys-35 in Trx) were primary targets for HNE modification. The covalent adducts formed between HNE and Trx were also confirmed by mass spectrum. Because the thioredoxin system is one of the core regulation enzymes of cells' function, inhibition of both TrxR and Trx by HNE provides a possibly novel mechanism for explanation of its cytotoxic effect and signaling activity, as well as the further damage indirectly caused under oxidative stress conditions.

Introduction

Lipid peroxidation is inevitably involved in normal cellular metabolism, and several stress causing agents, for example, oxidants, heat shock, UV irritation, and serum starvation, etc., will stimulate it to an uncontrolled high level. 4-Hydroxy-2-nonenal (HNE) is a major end aldehydic product, which derives from n-6 polyunsaturated fatty acids such as linoleic acid, linolenic acid, and arachidonic acid.[1] HNE is permanently formed at background levels in the physiological environment; however, its production is greatly enhanced under pathological conditions related to increased lipid peroxidation. It is generally agreed that a physiological concentration of HNE (below 1 μM) is involved in many signaling pathways, including protein kinase, mitogen-activated protein kinase, tyrosine kinase receptor, and so on, in cell growth, differentiation, and proliferation.[2] However, the concentration of HNE in pathological conditions, such as oxidative stress, can accumulate to more than 10 μM, or even as high as millimolar levels.[1,3,4] In such a case, HNE is toxic and can modify various cellular components such as protein and DNA.[5,6]

The thioredoxin system, composed of thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH, is the most important constituent of the intracellular redox milieu and is involved in many cellular signaling pathways.[7−9] It is ubiquitous from Archea to man. Mammalian TrxRs are unique large selenoproteins (M_r 114 000 or larger) containing a unique catalytically active selenolthiol/selenenylsulfide in the conserved C-terminal sequence -Gly-Cys-Sec-Gly.[10,11] Thioredoxins, from both mammalian cells and bacteria, are M_r 12 000 proteins with a conserved pair of cysteine residues (−CGPC−) in the active site, acting as the major disulfide reductase responsible for maintaining cytosolic proteins in their reduced state. The active site disulfide in oxidized Trx is subsequently reduced by electrons from NADPH via TrxR.[7−9]

HNE can modify many cellular targets especially those containing thiol groups (−SH),[1,4,6,12,13] such as glutathione, cysteine, glutathione S-transferase, and glutathione reductase. Mammalian TrxRs contain selenocysteine bearing a −SeH group, which is more sensitive than −SH group to alkylating agents.[14,15] In this study, we investigated the interaction between HNE and rat recombinant TrxR1 as well as Trx from *Escheri-*

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chia coli. We report herein that both of them can be irreversibly inhibited by HNE via modification of their catalytically active residues, that is, Cys-496/Sec-497 in TrxR and Cys-32/cys-35 in Trx, respectively. The inhibition of thioredoxin system by HNE provides a novel insight to understand the cytotoxicity of this reactive species as well as the indirect harmful effect caused by oxidative stress and lipid peroxidation.

Results and Discussion

HNE Can Irreversibly Inhibit TrxR in a Dose- and Time-Dependent Manner. When NADPH-reduced TrxR was incubated with HNE, its DTNB reduction and Trx-mediated NADPH oxidation activity were significantly lost with the IC_{50} value of around 3.8 μM (Figure 1). The HNE-induced inactivation of TrxR was also time-dependent. With longer incubation time, less activity of the enzyme remained (Figure 2). After removal of HNE from the incubation buffer by filtering through an Ultrafree-MC Millipore 30,000 cutoff filter, the enzyme activity could not be recovered, demonstrating that TrxR was irreversibly inhibited by HNE. Besides, by preincubation of oxidized TrxR with HNE and a following removal of the compound from the solution, the activity of the enzyme was not influenced (data not shown).

The Redox-Active Residues Cys496 and Sec497 Were Targets for HNE Modification. The reduced TrxR is an enzyme with high nucleophilicity because of its free −SH and −SeH groups, which are exposed to the surface of the enzyme,[11] and is vulnerable to attack by various alkylating agents.[14,15] Are the redox-active sites Cys496 and Sec497 targets for HNE? When TrxR was incubated with HNE prior to BIAM, which can alkylate free −SH and −SeH groups of Cys496 and Sec497, the blotting band intensity was weaker than the control (Figure 3). This result showed that those groups in TrxR were first alkylated by HNE. With the higher concentrations of HNE, the weaker band intensity can be observed. It has been reported that BIAM can selectively alkylate TrxR by adjusting the pH value.[15−17] In high pH value (pH 8.5), both −SH and −SeH groups were alkylated (lane 3 in Figure 3), but in low pH value (pH 6.5), only −SeH group was alkylated (lane 4), which is due to the low pK_a value for selenocysteine. When TrxR was treated with 50 μM HNE, most of the free −SH and −SeH groups were blocked (lanes 1 and 6). With even higher concentration of HNE (250 μM), there was no free −SH or −SeH group left (data not shown). This blotting result, coupled with the fact that HNE cannot inhibit oxidized TrxR, suggests that the redox-active residues of Cys496 and Sec497 in the enzyme are most likely targets for HNE covalent modification.

HNE Can Inhibit TrxR Dose-Dependently in HeLa Cells. To further study whether HNE can inhibit TrxR in vivo, we cultured HeLa cells with HNE and checked the TrxR activity in cell extracts. HNE can permeate the cell membrane easily because of its low molecular weight and lipid-solubility. No significant decrease of TrxR activity in cell extracts was observed when cells were cultivated with 10 μM HNE. However, with exposure of HeLa cells to 50 μM HNE for 6 h, the enzyme activity was remarkably lost as is clearly shown in Figure 4. This result demonstrates that HNE, at a relatively high concentration that can be easily reached under an oxidative stress environment, can inactivate TrxR in vivo.

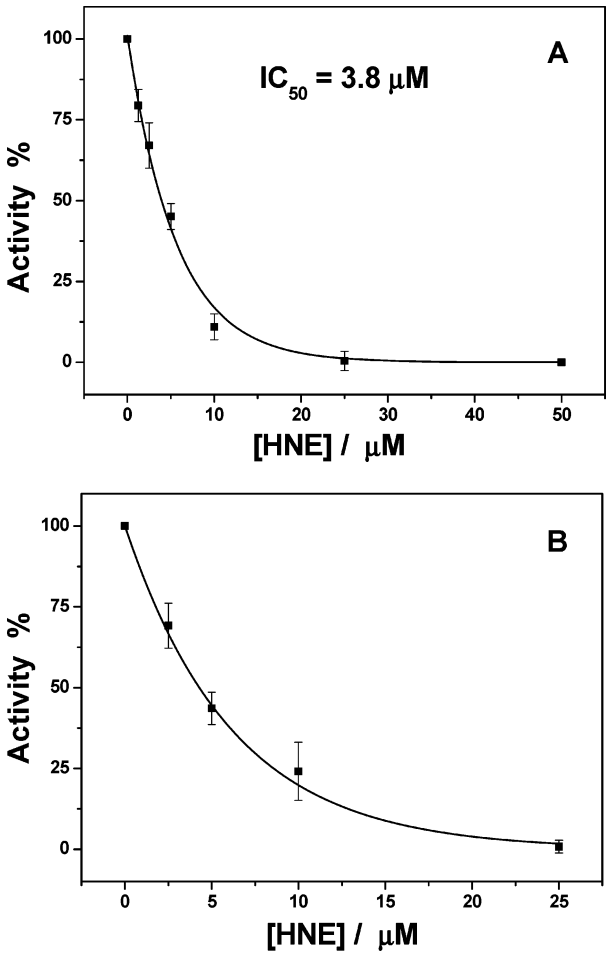

Figure 1. Dose-dependent inhibition of TrxR by HNE. (A) DTNB assay. A mixture of 0.2 μM TrxR and 100 μM NADPH in TE buffer was preincubated at room temperature for 5 min. Different concentrations of HNE were then added, and incubation was continued at room temperature for 2 h. The final concentration of ethanol was 1% (v/v), and the control had the same amount of ethanol. To assay the enzyme activity, the appropriate volume of the incubation mixture was taken out and added to a cuvette containing 2 mM DTNB and 200 μM NADPH. The increase of absorbance at 412 nm (A_{412}) was recorded immediately at the initial 1 min with a blank reference. The activity was expressed as the percentage of the control. (B) Trx-mediated insulin reduction assay. A mixture of 0.2 μM TrxR and 100 μM NADPH in TE buffer was preincubated at room temperature for 5 min. Different concentrations of HNE were then added, and incubation was continued at room temperature for 2 h. The final concentration of ethanol was 1% (v/v), and the control had the same amount of ethanol. To assay the enzyme activity, the appropriate volume of the incubation mixture was taken out and added to a cuvette containing 1 mg/mL insulin, 200 μM NADPH, and 10 μM *E. coli* Trx. The decrease of absorbance (A_{340}) was recorded immediately at the initial 1 min with a blank reference. The activity was expressed as the percentage of the control based on the rate of NADPH oxidation.

Trx Lost Its Ability To Reduce Insulin after Treatment with HNE. HNE can inactivate Trx in a dose- and time-dependent manner assayed by its ability to reduce insulin, which was shown in Figure 5. The activity of Trx cannot be rescued after removal of HNE, indicating that the inhibition is irreversible. There was no inhibition of oxidized Trx by this compound (data not shown). During incubation of Trx with HNE longer than 2 h, no further significant decrease of Trx activity was observed. This suggested that the interaction between reduced Trx and HNE reached equilibrium within 2 h. HNE can also

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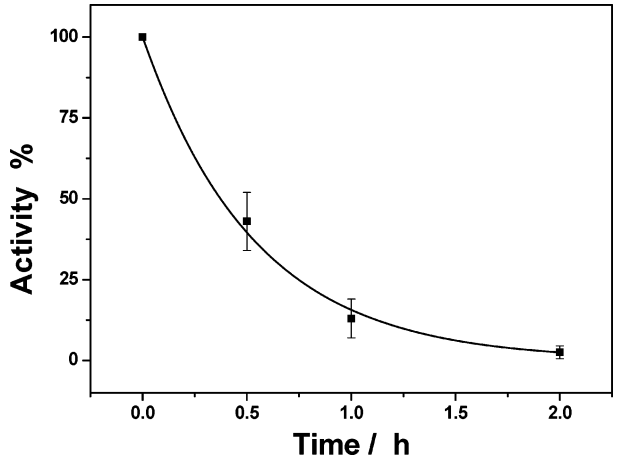

Figure 2. Time-dependent inhibition of TrxR by HNE. A mixture of 0.2 μM TrxR and 100 μM NADPH in TE buffer was preincubated at room temperature for 5 min. Next, 25 μM HNE was added, and incubation was continued at room temperature. The final concentration of ethanol was 0.5% (v/v), and the control had the same amount of ethanol. To assay the enzyme activity, 150 μL of the incubation mixture was taken out at different time intervals (0.5, 1, and 2 h) and added to a cuvette containing 2 mM DTNB, 200 μM NADPH in the total volume of 0.5 mL. The increase of A_{412} was recorded immediately at the initial 1 min with a blank reference. The activity was expressed as the percentage of the control. There was no influence of the enzyme activity after it was incubated with only ethanol at room temperature for 2 h.

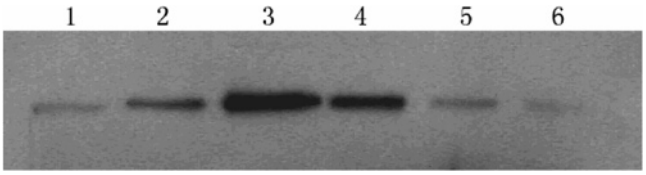

Figure 3. The redox-active residues Cys496 and Sec497 were targets for HNE modification. Different concentrations of HNE were added to NADPH-prereduced TrxR (0.9 μM) and incubated at room temperature for 2 h. The same amounts of ethanol were added to the control experiments. Lanes 1−3, alkylation of TrxR by BIAM at pH 8.5; lanes 4−6, alkylation of TrxR by BIAM at pH 6.5. Lanes 1 and 6, 50 μM HNE; lanes 2 and 5, 12.5 μM HNE; lanes 3 and 4, control.

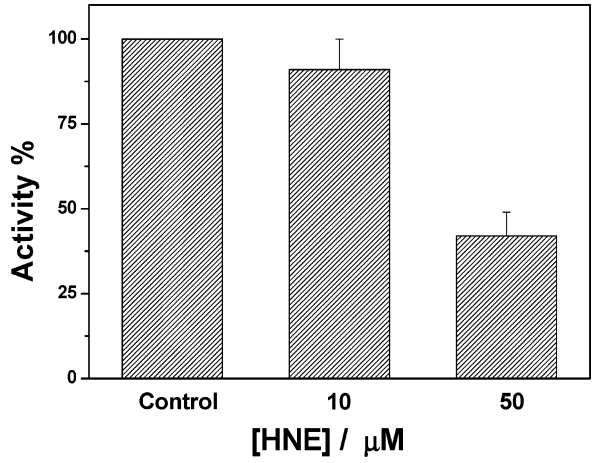

Figure 4. Inhibition of TrxR by HNE in HeLa cells. Cells were cultured in the presence of 10 or 50 μM HNE for 6 h, and the enzyme activity was measured and expressed as the percentage of the control, which contains the same amount of ethanol (1%).

inactivate wild-type human Trx1 in its reduced form with almost the same efficiency (data not shown).

Active Sites of Cys-32 and Cys-35 in Trx Were Targets for HNE Modification. Because HNE is a reactive intermediate

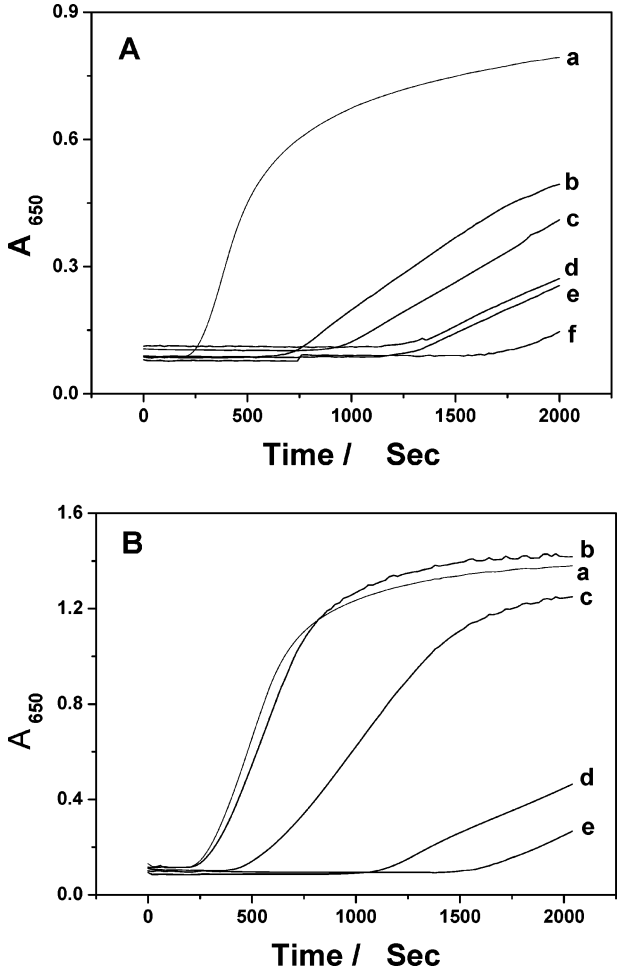

Figure 5. Inactivation of Trx by HNE. (A) Time-dependent inhibition of Trx by HNE. Prereduced Trx (4.5 μM) was incubated with 140 μM HNE anaerobically at 37 °C for different times. The final concentration of ethanol in the experiment was 0.2%, and the control contained the same amount of ethanol. The enzyme activity was measured at room temperature by monitoring the increase of A_{650}, which is caused by the precipitation of reduced insulin. [DTT] = 2 mM, [Trx] = 4 μM, and [insulin] = 0.8 mg/mL. (a) Positive control; (b−e) after incubation for 0.5, 1, 2, and 4 h; (f) negative control (without Trx). (B) Dose-dependent inhibition of Trx by HNE. Prereduced Trx (5 μM) was incubated with different concentrations of HNE anaerobically for 2 h at 37 °C. The final concentration of ethanol in the experiment was 1%, and the control contained the same amount of ethanol. The enzyme activity was measured at room temperature by monitoring the increase of A_{650}. [DTT] = 2 mM, [Trx] = 4 μM, and [insulin] = 1 mg/mL. (a) Positive control; (b−d) incubation with 10, 50, and 160 μM HNE; (e) negative control (without Trx).

produced during lipid peroxidation especially with high reactivity to thiol groups,[1,4,6,12] it was tested for Trx reaction. Preliminary results showed that it can irreversibly inhibit reduced Trx, which contains two −SH groups (Cys-32 and Cys-35), but not Trx in oxidized form. This suggests that the thiol groups in the protein might be the targets for HNE attacking. To further study the inhibition mechanism of Trx by HNE, we determined the number of thiol groups after Trx was treated with HNE. With the increase of the concentration of HNE, the free thiol groups in the protein decreased as is shown in Figure 6. After reduced Trx was incubated with a high concentration of HNE (600 μM), none of the free thiol groups were left. We also did a control experiment to evaluate if HNE interacts with 5-thiol-2-nitrobenzolic acid (TNB). Under our experimental concentrations, there is only a marginal effect (data not shown), which

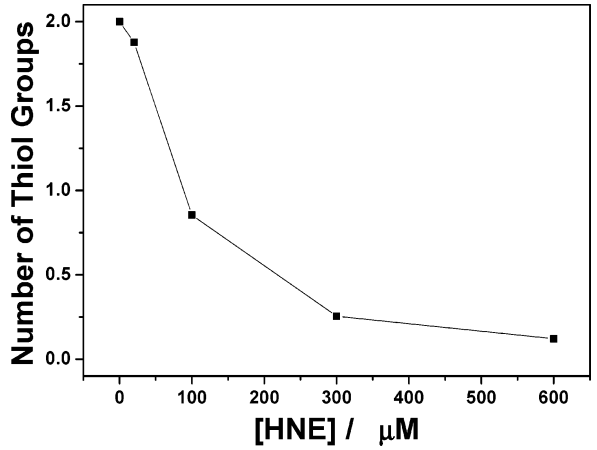

Figure 6. Loss of free thiol groups in Trx after treatment with HNE. The remaining free −SH groups in the reduced Trx after treatment with HNE anaerobically for 2 h at 37 °C were determined by titration with DTNB. The final concentration of ethanol in the experiment was 1%, and the control contained the same amount of ethanol.

Table 1. Analysis of the Mass Spectrum of HNE-Treated and Untreated Trx

mass of HNE-treated and untreated Trx	peak intensity (%)	mass difference	comments
11 665.67	100		untreated Trx
11 661.48	<5	−4.19 (∼0)[a]	remaining Trx in HNE-treated Trx
11 819.33		157.85 (∼156.2)	Trx + 1HNE
11 956.45		294.95 (2 × 156.4 − 18)	Trx + 2HNE − $1H_2O$
11 973.25	100	311.82 (2 × 155.9)	Trx + 2HNE
12 111.58		450.09 (3 × 156.0 − 18)	Trx + 3HNE − $1H_2O$
12 130.94		469.47 (3 × 156.5)	Trx + 3HNE

[a] This value is obtained by comparison to the mass of untreated Trx, that is, 11 665.67, while the others are compared to the mass of remaining Trx in HNE-treated Trx, that is, 11 661.48.

excluded the possibility that the decrease of absorbance at 412 nm during DTNB-titration was due to the reaction between HNE and TNB. These results suggested that the inhibition is caused by modification of the thiol groups in Trx. To confirm the titration results, we compared the molecular weight of Trx treated with or without HNE by mass spectrum. It is clearly seen from Figure 7 that Trx had the expected molecular weight of 11 665.67 and, after incubation with HNE, besides the weakly remaining peak of unmodified Trx (indicated by the arrow in Figure 7), induced formation of several new peaks, which correspond to different modification of Trx by HNE. The detailed analysis of the mass difference between the treated and untreated Trx is listed in Table 1.

The thioredoxin system is the most important constituent of the intracellular redox milieu. Mammalian TrxRs, much distinct from those from plants or bacteria, are multifunctional homodimeric selenocysteine-containing enzymes with a broad substrate specificity besides their native substrate Trxs.[9,18−20] However, mammalian and *E. coli* Trx's share a common Trx-fold with the same active site (−CGPC− and reactivity toward disulfides.[7,9] The thiol−disulfide exchange reactions are rapid and reversible, which are suited to control protein function via the redox state of structure or catalytic −SH groups. This mechanism of thiol redox control is of physiological importance in many crucial processes such as resistance against oxidative stress, ribonucleotide reduction, and transcription factor modulation.[7−9] The Trx/TrxR system is a major ubiquitous disulfide reductase responsible for maintaining intracellular proteins in their reduced states.

Our results demonstrate that HNE can only inactivate TrxR and Trx in their reduced form. In the reduced form of TrxR, which should be the major form present in cells with the presence of NADPH, the redox-active sites of Cys496 and Sec497 are present in the form of free −SH/−SeH and exposed to the surface of the enzyme,[11] which makes them easily attacked by alkylating agents.[14,15] Yu et al. reported recently that HNE can form a covalent adduct with TrxR by immunochemical analysis.[21] Our blotting results further supported that both of the redox residues of Cys496 and Sec497 in active site were modified when the enzyme was incubated with HNE. The α,β-unsaturated aldehyde structure in HNE makes it act as a potent alkylator. There is an equilibrium between HNE and its enol form, and the latter is more sensitively attacked by nucleophilic agents. The presence of a 4-hydroxy group can further stabilize the carbon cation in the enol form of HNE due to the inductive effect.[6] The pK_a value for selenocysteine is 5.1,[22] so it is clear that the selenocysteine in the enzyme is present in the predominant form of selenide ($-Se^-$) in our experimental conditions (pH 7.5). The selenide, with high nucleophilicity and exposed to the surface of the enzyme, can attack the carbon cation in the enol form of HNE effectively and produce the Michael adduct. It is the same mechanism for the −SH group to react with HNE. A proposed mechanism for HNE to inactivate TrxR is shown in Scheme 1. There are two free −SH groups in reduced *E. coli* Trx. Through the combination of titration result and mass spectrum, we provided that both of the free −SH groups, Cys32 and Cys35, were targets for this compound. It is the same inhibition mechanism as that of TrxR by HNE, that is, forming a covalent adduct by alkylating both thiol groups by HNE. The less reactivity of the −SH group than the −SeH group can explain why it needs a relatively higher concentration of HNE to inhibit Trx than TrxR in vitro.

Other studies indicated that, besides cysteine, histidine and lysine residues can also be alkylated by HNE to form a Michael adduct.[4,23] In the mass spectrum of HNE-treated Trx (Figure 7), the molecular weight of 12 130.95 which is caused by the addition of three molecules of HNE to Trx, should be due to the reaction of histidine or lysine residues with this electrophilic species. The Michael adduct for HNE can rearrange to a cyclic hemiacetal via reaction of the 4-hydroxyl group with the aldehyde and subsequently losing one molecule of H_2O.[5,6] Based on this point, it is reasonable to accept the observed molecular weights of 11 956.43 and 12 111.57 in HNE-treated Trx. The order for reactivity of protein residues toward HNE was found

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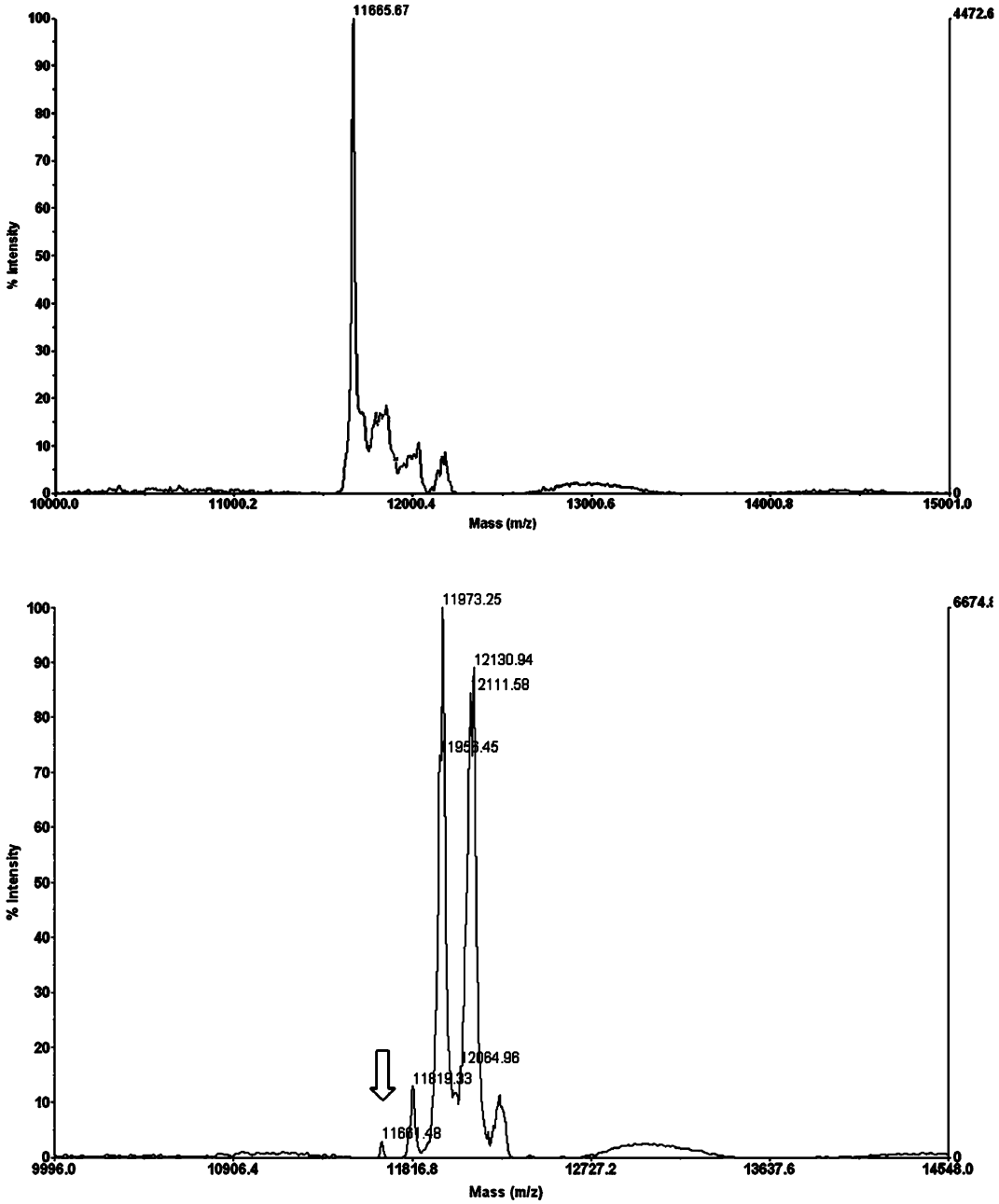

Figure 7. MALDI-TOF mass spectrum of HNE-treated and untreated Trx. For the detailed analysis of the spectrum, please refer to Table 1.

to be cysteine ≫ histidine > lysine.[5] This makes cysteine a target with priority for HNE modification.

The IC_{50} value of 3.8 μM for TrxR is in the low range of values that were measured for other different enzymes activity.[4,24,25] HNE can inactivate protein disulfide isomerase,[26] inhibit proteasome,[27] and induce hematopoietic stem cells apoptosis.[28] Recently, Carbone et al. reported that HNE can inhibit Hsp-72-mediated protein refolding,[29] and Feng et al. reported inhibition of nucleotide excision repair by HNE.[3] All of these results, coupled with our findings that HNE can inactivate the thioredoxin system, make this reactive compound a potent toxic agent. HNE is a reactive compound and can interact with a variety of intracellular components. Although there are several metabolic pathways for HNE in cells,[6,12,25] such as to form 4-hydroxynonanal catalyzed by alkenal/one oxidoreductase, to form 4-hydroxynonenoic acid catalyzed by aldehyde dehydrogenase, to form 4-hydroxynonenol catalyzed by alcohol dehydrogenase, to form glutathione-HNE adduct catalyzed by glutathione S-transferase, and so on, our result that HNE can inhibit TrxR in HeLa cells proved that this inhibition does occur in vivo. We previously found that curcumin was also an irreversible inhibitor of TrxR with an IC_{50} value of 3.6 μM in vitro,[15] almost the same as HNE. However, HNE is a weaker inhibitor than curcumin in HeLa cells. As was motioned above, the high reactivity and many metabolic pathways in vivo

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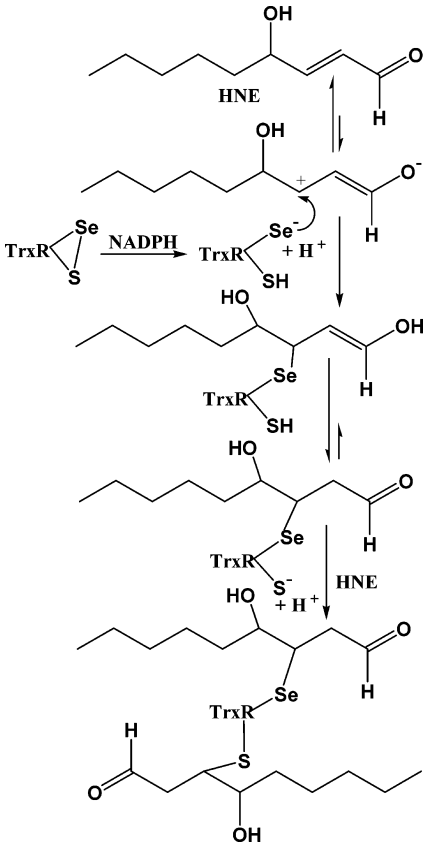
Scheme 1. Proposed Mechanism for Irreversible Inhibition of TrxR by HNE



will lead to a low availability of HNE to TrxR, thus lowering its inhibition effect.

Oxidative stress occurs when cells are exposed to elevated levels of reactive oxygen species (ROS), which is inevitably produced in normal cellular metabolism. ROS can attack various intracellular components, such as DNA, proteins, and lipids. Because ROS-induced lipid peroxidation is a free radical chain reaction and one initial free radical can produce more than 20 propagating radicals,[30] it makes lipids vulnerable targets for ROS. This can explain why HNE, a major end product of lipids peroxidation, is permanently present in low concentration (less than 1 μM) even in normal physiological conditions. However, under oxidative stress conditions, HNE can accumulate to a higher concentration, as high as millimolar level, and becomes toxic. The thioredoxin system is one of the core enzymes in all of the cells. Inhibition of the system can change many intracellular signaling pathways. For example, inhibition of TrxR/Trx will activate apoptosis signaling kinase-1 (ASK-1), which plays essential roles in apoptotic regulation and is regulated by redox state of Trx.[31] Second, the thioredoxin system is a key enzyme to maintain the intracellular reduced environment and can act as a direct antioxidant by scavenging ROS[32] or indirect antioxidant by regenerating other antioxidants[9,18,19,33,34] and serving as an electron donor to other enzymes, such as peroxiredoxins and methionine sulfoxide reductase, to resist oxidative stress.[35,36] All of these functions will be lost by inhibition of TrxR/Trx and lead to oxidized intracellular environment. The ability of HNE to inactivate thioredoxin system, a central antioxidant enzyme, suggests that oxidative stress and lipid peroxidation may initiate a positive feedback loop that enhances the potential for oxidative damage. The produced oxidative stress can also activate ASK-1 and several other signaling pathways, and subsequently causes cell apoptosis via redox signaling pathways. Third, inhibition of TrxR/Trx will lose one major electron-donating pathway to ribonucleotide reductase, which is a key enzyme to reduce ribonucleotides to deoxyribonucleotides for DNA synthesis. Also, it was reported by Anestal et al. recently that TrxR modified by alkylating agents can directly induce cell apoptosis.[37] Taking together the above facts, it is no wonder that inhibition of the system by HNE should contribute positively to the cytotoxicity of this compound. The cascade effects after inhibition of Trx/TrxR were discussed in detail in our previous paper.[15]

Conclusion

In summary, we have herein characterized the irreversible inhibition mechanism of TrxR and Trx by HNE. The inhibition was caused by covalent modification of active residues in catalytic sites, that is, Cys496/Sec497 in TrxR and Cys32/Cys35 in Trx. Both of the inhibitions are redox state-dependent, and only the reduced enzymes can be inactivated. Because the thioredoxin system is a key enzyme to regulate a variety of redox-related cellular functions and HNE is permanently present in vivo and can accumulate to a much higher concentration under several pathological states, this interaction between the thioredoxin system and HNE gives us further insight to understand the cyototoxic effect of this compound and the indirect damage caused by oxidative stress.

Materials and Methods

Chemicals and Enzyme. Recombinant rat TrxR1 was essentially prepared as described previously.[38] The enzyme was pure as judged by Commassie-stained SDS-polyacrylamide gel electrophoresis (PAGE) and had a specific activity of 50% ($\sim$33 s^{-1}) of wild thioredoxin reductase with DTNB assay.[39] Trx from *Escherichia coli* was a homogeneous preparation as described by Ren et al.[40] NADPH, guanidine hydrochloride, bovine serum albumin (BSA), dithiothreitol (DTT), insulin, and DTNB were from Sigma. Biotinconjugated iodoacetamide (BIAM) was from Molecular Probes. BIAM was dissolved in 0.2 M Tris-Cl, 1 mM EDTA, pH 6.5 and 8.5, respectively. HNE was obtained from Calbiochem as a stock solution in ethanol (10 mg/mL) and was kept at − 80 °C. All other reagents were of analytical grade.

Enzyme Activity Assays. The activity of enzyme was determined at room temperature using a thermostatic Ultrospec UV/visible spectrophotometer (Shimadzu, CPS-260) in a buffer containing 50 mM Tris-Cl, 1 mM EDTA, pH 7.5 (TE buffer). TrxR was first reduced by incubation with excess NADPH at room temperature for 5 min.

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Appropriate amounts of HNE were then added to the NADPH-reduced enzyme followed by incubation at room temperature for the appropriate time. The same amounts of ethanol were added to the control experiments. The enzyme activities were measured by DTNB and Trx-mediated insulin reduction assays according to the available protocols.[39]

Detection of the HNE-Modified Residues in TrxR. Different concentrations of HNE were added to prereduced TrxR (0.9 μM), which was incubated at room temperature for 2 h. The same amounts of ethanol were added to the control experiments. After incubation, 1 μL of the reaction mixture was taken out and added to new tubes containing 19 μL of 100 μM BIAM (pH 6.5 and 8.5, respectively), following incubation at 37 °C for another 30 min to alkylate the remaining free −SeH and −SH groups in the enzyme.[16,17] Ten microliters of BIAM-modified enzyme was mixed with 20 μL of loading buffer, 20 μL of the samples was subjected to SDS-PAGE on a 7.5% gel, and the separated proteins were transferred to nitrocellulose membrane. Proteins labeled with BIAM were detected with horseradish peroxidase (HRP)-conjugated streptavidin and enhanced chemiluminescence (ECL) detection.

Inhibition of TrxR by HNE in HeLa Cells. HeLa cells were cultivated in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% heat inactivated FBS (PAA, Pasching, Austria), 2 mM glutamine (PAA), and 100 units mL^{-1} penicillin/streptomycin (PAA). The cells were cultured at 37 °C in an incubator with 90% humidified atmosphere containing 5% CO_2. HeLa cells were incubated with different concentrations of HNE for 6 h in an incubator. The control group contained the same amount of ethanol (1%, V/V). Before harvesting, we washed cells with phosphate-buffered saline (PBS), and then lysed cells with cell lysis buffer (0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl in TE buffer) in the presence of protease inhibitors (complete, EDTA-free, 1873580, Roche). The activity of TrxR in the cell extracts was determined as described elsewhere.[39] Protein concentration was quantified employing the Bio-Rad procedure using BSA as a standard. A volume corresponding to 38 μg of protein from each cell extract was incubated with TE buffer containing 400 μM NADPH, 2 mg/mL insulin, and 14 μM *E. coli* Trx at room temperature for 30 min in a final volume of 50 μL. By the addition of 500 μL of 1 mM DTNB in 6 M guanidine hydrochloride (pH 8.0), the reaction was terminated. A blank sample, containing everything except Trx, was treated in the same manner. The absorbance at 412 nm was measured, and the blank value was subtracted from the corresponding absorbance value of the sample. The activity of the enzyme was expressed as the percentage of the control.

Inactivation of Trx by HNE. Trx−$(SH)_2$ was prepared from *E. coli* Trx-S_2 by incubation at room temperature for 20 min with 100 mM DTT. DTT was subsequently removed by gel chromatography on a Sephadex G-25 column using N_2-equilibrated TE buffer. The appropriate amounts of HNE were then added to the prereduced Trx following incubation at 37 °C anaerobically for the appropriate time. The same amounts of ethanol were added to the control experiments. The Trx activities were measured by its ability to reduce insulin in the presence of DTT according to the available protocols.[39]

Titration of Trx Free Thiol Groups with DTNB. The remaining free −SH groups in the reduced Trx after treatment with HNE were determined by titration with DTNB by the method of Ellman[41] with a little modification. Briefly, 25 μM Trx$(SH)_2$ was incubated with different concentrations of HNE (0−600 μM) anaerobically for 2 h at 37 °C. After incubation, 1 mM DTNB in 6 M guanidine hydrochloride, pH 8.0, was added, and the absorbance at 412 nm was read against the reference containing the same amount of DTNB.

Mass Analysis of Adducts Formed between HNE and Trx. One hundred microliters of reduced Trx (40 μM) was incubated with 5 μL of 5 mM HNE at 37 °C for 2 h. Before mass analysis was performed, 0.5 mM DTT was added to keep the protein in its reduced form and block the excess HNE. The molecular weights of Trx and the adducts were recorded with MALDI mass spectrometry at PAC, the core facility at Karolinska Institute (Applied Biosystems).

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