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Systems Analysis of Protein Modification and Cellular Responses Induced by Electrophile Stress

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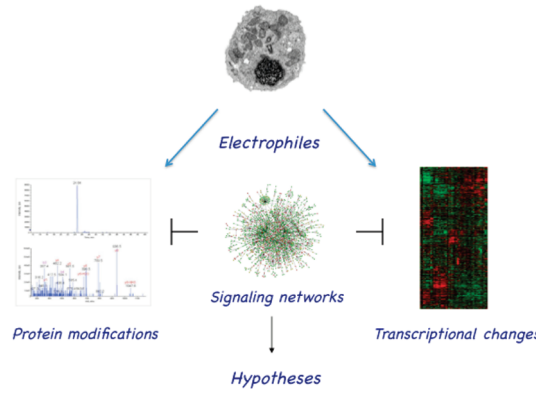

Biological electrophiles result from oxidative metabolism of exogenous compounds or endogenous cellular constituents, and they contribute to pathophysiologies such as toxicity and carcinogenicity. The chemical toxicology of electrophiles is dominated by covalent addition to intracellular nudeophiles. Reaction with DNA leads to the production of adducts that block replication or induce mutations. The chemistry and biology of electrophile—DNA reactions have been extensively studied, providing in many cases a detailed understanding of the relation between adduct structure and mutational consequences. By contrast, the linkage between protein modification and cellular response is poorly understood.

In this Account, we describe our efforts to define the chemistry of protein modification and its biological consequences using lipid-derived α,β-unsaturated aldehydes as model electrophiles. In our global approach, two large data sets are analyzed: one represents the identity of proteins modified over a wide range of electrophile concentrations, and the second comprises changes in gene expression observed under similar conditions. Informatics tools show theoretical connections based primarily on transcription factors hypothetically shared between the two data sets, downstream of adducted proteins and upstream of affected genes. This method highlights potential electrophile-sensitive signaling pathways and transcriptional processes for further evaluation.

Peroxidation of cellular phospholipids generates a complex mixture of both membrane-bound and diffusible electrophiles. The latter include reactive species such as malondialdehyde, 4-oxononenal, and 4-hydroxynonenal (HNE). Enriching HNE-adducted proteins for proteomic analysis was a technical challenge, solved with click chemistry that generated biotin-tagged protein adducts. For this purpose, HNE analogues bearing terminal azide or alkyne functionalities were synthesized. Cellular lysates were first exposed to a single type of HNE analogue (azido- or alkynyl-HNE), and then click reactions were performed against the cognate alkynyl- and azido-biotin derivative. The resulting biotin-labeled proteins were captured and enriched over a streptavidin matrix for subsequent mass spectrometric analysis. We thereby identified a multitude of HNE targets. Simultaneous microarray analysis of changes in gene expression triggered by HNE also produced an abundance of data. Functional analysis of both data sets generated the hypothesis that an important pathway of cellular response derives from electrophile modification of protein chaperones, resulting in the release of transcription factors that are their clients. Informatic analysis of the protein modification and microarray data sets identified several transcription factors as potential mediators of the cellular response to HNE-adducted proteins. Among these, heat shock factor 1 (HSF1) was confirmed as a sensitive and robust effector of HNE-induced changes in gene expression. Activation of HSF1 appears, in part, to be mediated by the electrophilic adduction of Hsp70 and Hsp90, which normally maintain HSF1 in an inactive cytosolic complex.

The identification of HSF1 as a mediator of biological effects downstream of HSF1 has provided new opportunities for research, illustrating the potential of our systems-based approach. Accordingly, we characterized HSF1-mediated gene expression in protecting against electrophile-induced toxicity. Among the genes induced by HSF1, Bd-2- associated athanogene 3 (BAG3) is notable for its actions in promoting cell survival through stabilization of antiapoptotic Bd-2 proteins, appearing to have a critical role in mediating cellular protection against electrophile-induced death.

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Introduction

Cells respond to a diverse array of environmental and endogenous stimuli through cell surface and intracellular receptors. Once engaged, these receptors trigger signaling cascades that culminate in a cellular response. These communication systems represent networks developed through evolution that enable cellular adaptation to the surrounding environment. In addition to these receptor-mediated pathways, cells can also respond to nonspecific challenges presented by reactive oxidants and electrophiles. The structures and comparative reactivities of these agents are highly diverse, so as a general rule specific receptors for reactive intermediates have not evolved. Rather, generic mechanisms exist that mediate cellular responses to chemical stress. The signaling pathways activated by these reactive species either enhance cytoprotective processes or trigger cell death in the case of overwhelming stress.

Electrophiles represent a significant threat to cellular law and order because they react with a multitude of intracellular nucleophiles including DNA, RNA, phospholipids, and proteins. Electrophiles are generated during the enzyme-mediated oxidation of foreign compounds (toxicants, foods, pharmaceuticals), as well as by the oxidation of endogenous biomolecules (lipids, amino acids, carbohydrates). Much of our knowledge regarding the biochemistry of electrophiles comes from extensive research into their role in carcinogenesis. Decades of work have addressed various aspects of electrophile stress within this context and have helped to define the mechanisms for electrophile generation from various sources; the covalent modification of DNA bases; the biochemical mechanisms of mutagenesis and repair; and the correlation between electrophilic DNA adducts and cellular transformation (Figure 1). These investigations have been aided by a powerful approach in which single DNA adducts are incorporated into a viral genome or shuttle vector, and the fates of the adducts are subsequently evaluated in intact cells.[1] This method has provided direct correlations between the structures of DNA adducts, their metabolism in vivo, and their mutagenic potential.

Less effort has been devoted to the study of macromolecular targets other than DNA. However, it is increasingly apparent that they represent important sites for electrophilic adduction with respect to both the degree and the biological significance of their modification.[2] In addition, there is a growing appreciation that cells are not passive targets for electrophilic damage but can adapt and protect themselves from subsequent stress. Much of this heightened interest derives from improved technologies that have enabled researchers to better address the role of macromolecular modification (especially protein modification) in sensing and mediating biological responses to reactive species. For example, the reactions between electrophiles and amino acid side chains are relatively well-defined, and techniques exist for detecting electrophilic modifications to individual peptides or proteins.[3] However, establishing a definitive link between the adduction of a specific protein and a biological response has proven to be a more challenging task. Some progress has been made by examining proteins that participate in well-defined signaling pathways and addressing the effect of their modification on the function of the signaling network. For example, modification of IκB kinase by the reactive electrophiles $\Delta^{12,14}$-15-deoxy-PGJ$_2$, 4-hydroxynonenal (HNE), or parthenolide blocks activation of the NF-κB signaling pathway[4−6] (Figure 2). Modification of IκB kinase interferes with the release of NF-κB subunits from IκB and attenuates NF-κB-mediated gene expression.[4] Valuable information has been gleaned from such focused investigations, but the pace of discovery has been slow. Moreover, the relative importance of individual signaling pathways within the context of the overall cellular response is difficult to assess in this manner. Recent advances that enable large sets of modified proteins to be identified, combined with global analyses of gene expression, have provided an opportunity for a systems-based approach to study electrophile stress at the cellular level. We will describe our initial attempts to construct such an approach within the context of our ongoing investigations, systematically examining the cellular responses to electrophiles generated from oxidized lipids.

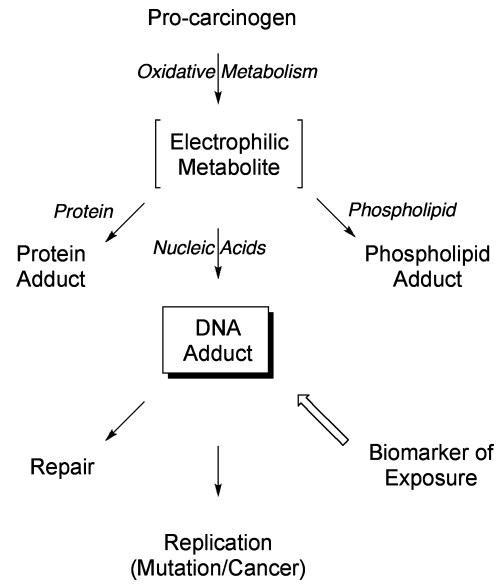

FIGURE 1. Role of electrophile generation in cellular damage and carcinogenesis.

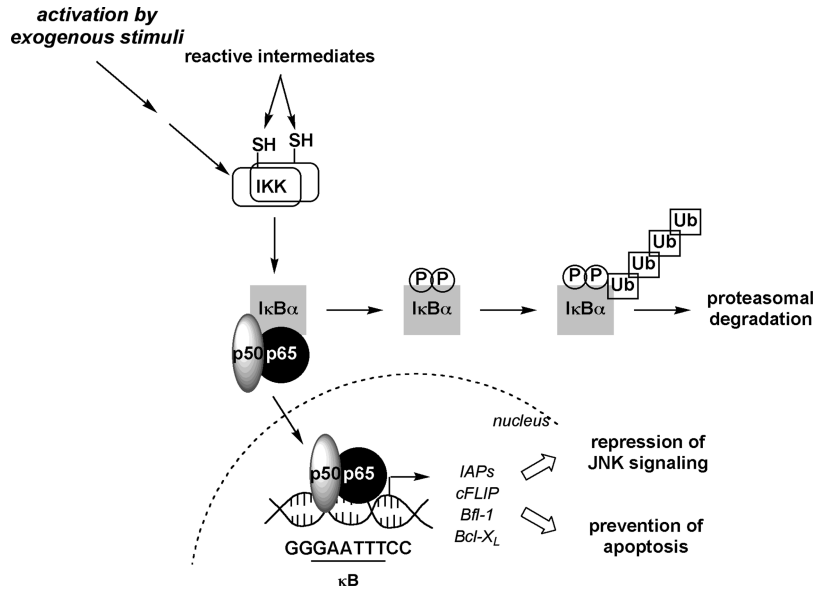

FIGURE 2. Inhibition of NF-κB signaling by reactive electrophile modification of IκB kinase.

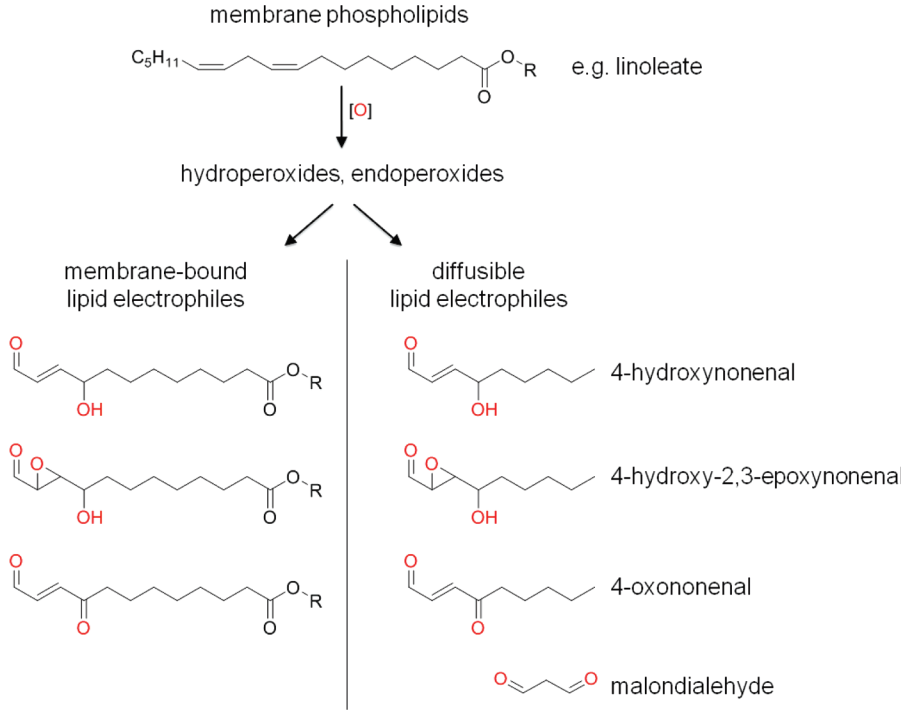

FIGURE 3. Generation of membrane-bound and diffusible electrophiles from glycerophospholipid peroxidation.

Oxidative Stress and Lipid Electrophile Generation

Research in our laboratory has focused on electrophiles generated as a result of glycerophospholipid peroxidation, the spontaneous oxidation of the unsaturated fatty acyl side chains esterified to the glycerol backbone (Figure 3). Oxidants generated by a variety of pathways remove *bis*-allylic hydrogen atoms to generate pentadienyl radicals that are scavenged by O_2 to form lipid peroxyl radicals. The lipid peroxyl radicals remove a hydrogen atom from a neighboring polyunsaturated fatty acid residue to propagate the radical chain and produce a fatty acyl hydroperoxide bound to the phospholipid.[7] The *sn*-2 position of all membrane glycerophospholipids consists of mono- or polyunsaturated fatty acids, so the potential for lipid peroxidation is enormous. In fact, by monitoring certain products of lipid peroxidation as their urinary metabolites, it has been unequivocally established that phospholipid peroxidation occurs continuously in humans and can be increased by oxidant challenges such as cigarette smoking or xenobiotic exposure.[8] Reduction of the initially formed

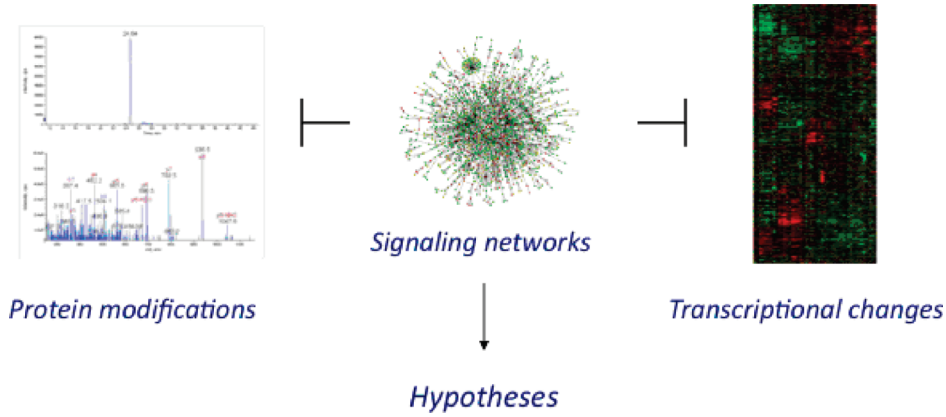

FIGURE 4. Approach to relating electrophile modification of proteins to transcriptional responses. Inventories of modified proteins and changes in gene expression are determined in parallel experiments. Potential linkages between the two data sets are established by informatic analysis of signal transduction networks. This allows formulation of hypotheses relating modification of individual proteins to altered gene expression.

fatty acyl hydroperoxides by one-electron reductants generates alkoxyl radicals that decompose to a plethora of products, some of which contain reactive functional groups such as epoxides and aldehydes. Depending on the chemistry of hydroperoxide decomposition, fragmentation products can be produced in which the electrophile is released and diffuses throughout the cell or in which the electrophile remains bound at the *sn*-2 position of the phospholipid.

We are particularly interested in the chemical biology of α,β-unsaturated aldehydes, such as malondialdehyde and HNE. Research from several groups, including our own, has established that these electrophiles react with DNA to generate mutagenic adducts that are detectable even in the genomes of healthy individuals.[9] In addition to DNA, malondialdehyde and HNE react with proteins. Lipid-derived adducts to protein are detectable in mammalian tissue and can alter the properties of the target proteins.[2] The reactivity of HNE within the cell is affected by such diverse factors as pH, glutathione content, and protein concentration, which together influence the complement of adducted proteins and degree of modification. Until recently, our understanding of HNE biology was mainly derived from the study of individual target proteins on a case-by-case basis. For example, the contribution of HNE to pathological events such as neurodegeneration, pain, inflammation, and cellular aging has largely been defined through the study of individual protein targets.[5,10–12]

Systems Analysis of Electrophile Stress

We have initiated a program to relate protein modification by electrophiles to cellular responses in a global fashion. The approach is outlined in Figure 4. Cells are treated with an electrophile (in this case HNE), and two large data sets are generated. The first is an inventory of the proteins modified by HNE, and the second is a compilation of the gene expression changes determined by microarray analysis. Transcriptional activation or inhibition is not the only response of a cell to stress, but it integrates many changes in signaling networks that culminate in an ultimate cellular outcome. These protein modification and gene expression data sets are then linked by informatic analysis of the signaling networks engaged. For example, by monitoring changes in gene expression, one can infer which transcription factors are either activated or inhibited during the cellular response to electrophile treatment. Extrapolating data in this manner can help to identify which signaling networks are affected by the electrophile. The next step is to examine protein modification data, to ask if one can plausibly connect a HNE-modified protein to the affected signal transduction pathways. This enables one to formulate hypotheses linking protein modification to transcriptional response that can be tested using molecular biological and biochemical approaches (e.g., reporter-based assays of gene expression, siRNA knockdown, etc.). Major challenges presented by this approach include developing the capture chemistry (the method by which the modified proteins are enriched for subsequent identification) and bioinformatic analysis (the tools used to link protein modification to transcriptional changes).

Reaction of HNE with proteins occurs primarily by Michael addition to histidine, cysteine, and lysine residues (Figure 5).[13] The initial Michael adducts cyclize to hemiacetals that have the capacity to cross-link lysine residues. HNE also reacts with lysine to form a Schiff base, but this is quantitatively less significant than Michael addition. The Schiff base can cyclize and dehydrate to form a stable pyrrole adduct.[14] In a highly oxidizing cellular environment, protein cross-linking reactions involving histidine or lysine residues has also been demon-

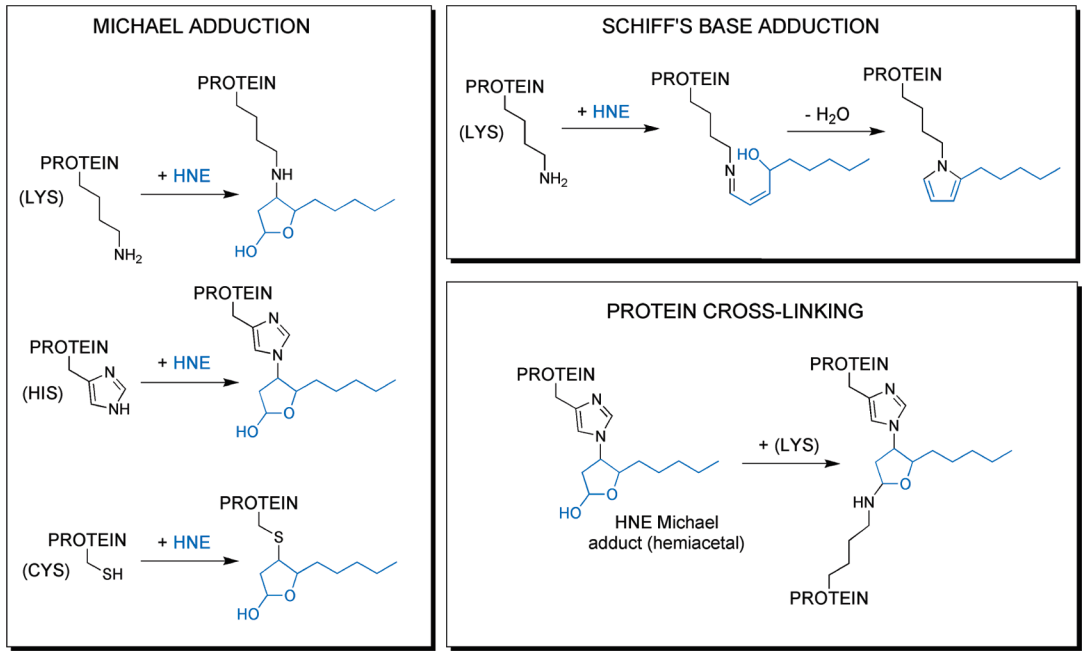

FIGURE 5. HNE reactions with amino acid side chains.

strated to arise from the bifunctional generation of both Michael and Schiff base adducts. Until recently, the identification of HNE-modified proteins relied primarily on immunochemical methods for adduct capture or detection. These methods are powerful, but they have significant limitations. Many antibodies are raised against specific HNE-amino acid adducts, so they may not recognize a broad range of adducts, whereas some antibodies may cross-react with proteins or adducts generated by oxidants or electrophiles other than HNE.

Proteomic Profiling of HNE Modification of Proteins

In order to supersede the use of antibody-based methods, we sought an approach that would be specific for HNE-modified proteins and would capture HNE adducts regardless of their chemical structure. Biotin hydrazide has been used to derivatize proteins modified by carbonyl-containing lipid oxidation products for enrichment with avidin-based matrices.[15−17] Although biotin hydrazide reacts with free protein aldehydes, it does not react with HNE adducts derived from the lysine Schiff base and it can react with adducts derived from other aldehydes and ketones.[18]

We utilized click chemistry to biotin-label HNE-modified proteins.[19] Click chemistry describes the 1,3-dipolar cycloaddition reaction between azide (or alkyne) labeled probes to conjugate alkyne (or azide) labeled reporter tags. This approach has proven extremely useful for interrogating biochemical targets in the complex environment of the cell.[20] For our purposes, the probe is an analogue of HNE, modified at the terminal carbon with an azide (azido-HNE) or substituted at the ω and ω-1 positions with an alkyne (alkynyl-HNE)[19] (Figure 6). The attachment of either an azide or alkyne tag to HNE is a subtle change so that HNE, azido-HNE, and alkynyl-HNE exhibit comparable cytotoxicity and abilities to stimulate gene expression (Figure 6). We used click chemistry, streptavidin-based enrichment, and mass spectrometry to compile large data sets of protein targets of azido-HNE or alkynyl-HNE in the human colon cancer cell line, RKO.[19] Individual proteins were validated as HNE targets based on the concentration−response to HNE analogues, as well as on the specificity of their modification. Protein modification data were then used in conjunction with results from gene expression studies to generate hypotheses on the signaling processes affected by HNE and the resulting cellular consequences.

HNE Induction of Gene Expression

We examined the effects of HNE on gene expression in the RKO cell line under conditions that were similar to those used to evaluate protein modification, varying both concentration and time of compound exposure.[21] Changes in global transcript levels were monitored using microarray techniques. Clustering genes into groups regulated by a common signaling pathway proved useful in characterizing the cellular response to HNE. For example, mapping transcriptional changes based on upstream regulatory pathways revealed activation of the DNA damage (HDM2; TP53INP1), antioxidant (HMOX1; SCL3A2; GCLM; NQO1), ER stress (ASNS; CTH;

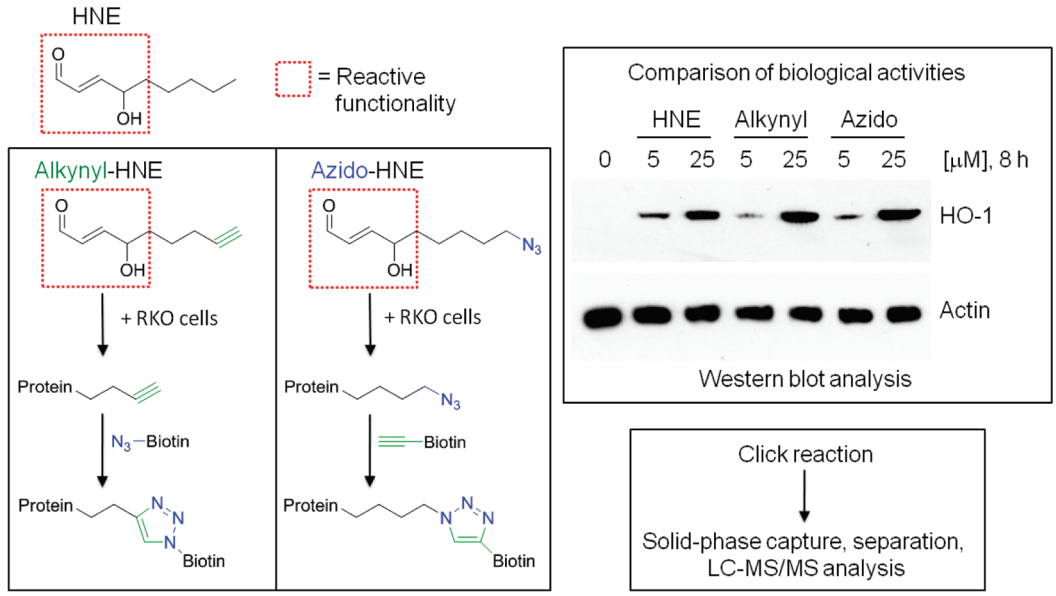

FIGURE 6. Method for adduct capture and analysis utilizing click chemistry compatible HNE analogues.

ATF3; GADD154), and heat shock (HSPA1A; HSPB1; HSPH1; DNAJB1) responses (Table 1). Activation of these pathways was individually confirmed using real-time PCR, Western blot analysis, and luciferase reporter assays.

We used a global approach to relate microarray and protein adduction data in a more directed manner. This was achieved using various software tools for sorting and mining both protein modification and gene expression data sets. For example, WebGestalt, which stands for "WEB-based Gene SeT AnaLysis Toolkit", is a program that integrates information from various public resources, uncovering genetic and biochemical relationships in complex data sets.[22] We have also employed GenMAPP, which facilitates the analysis of microarray data within the context of biochemical processes and human disease.[23] The results of these analyses are summarized in Figure 7. The dots at the bottom of this diagram represent the various genes that are upregulated (red) or downregulated (green) in response to HNE. Progressing upstream, these are connected by lines to the *cis* regulatory elements that control their expression. The *cis* regulatory elements are in turn connected by lines to transcription factors that bind them. A final set of lines are drawn from the transcription factors to the data set of HNE-modified proteins, representing connections via direct interactions or signal transduction pathways between a modified protein and the transcriptional regulators it affects. These connections establish plausible literature-based relationships between protein modification and the cellular response of transcriptional activation and enable the formulation of testable hypotheses.

TABLE 1. Gene Ontology Report of Biological Processes Based on Changes in Gene Expression (greater than 1.5-fold up- or downregulated) in HSF1 siRNA versus Control siRNA-Treated Cells Challenged with 45 μM HNE for 6 h

GO TERM (biological processes)	genes changing (>1.5-fold)	genes measured	% changed (>4.0%)
nucleosome assembly	18	106	17
chromatin assembly or disassembly	20	152	13.2
negative regulation of transcription	11	116	9.5
protein complex assembly	22	242	9.1
negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	11	126	8.7
establishment or maintenance of chromatin architecture	20	232	8.6
negative regulation of cellular metabolism	12	148	8.1
protein folding	15	222	6.8
apoptosis	17	255	6.7
regulation of protein kinase activity	7	110	6.4
unfolded protein binding	10	159	6.3
negative regulation of cell proliferation	8	129	6.2
DNA repair	11	187	5.9
response to DNA damage	12	206	5.8
IκB kinase NF-κB cascade	6	105	5.7
response to endogenous stimulus	12	219	5.5
negative regulation of apoptosis	5	102	4.9
cell surface receptor linked signal transduction	6	123	4.9
regulation of cell size	6	123	4.9
DNA replication	7	146	4.8
regulation of cell proliferation	12	252	4.8
steroid metabolism	6	129	4.7
protein transport	9	196	4.6
humoral immune response	7	160	4.4
ubiquitin cycle	11	251	4.4
histogenesis	6	142	4.2
muscle development	6	142	4.2
growth	6	127	4.0

The use of bioinformatics tools greatly facilitated the discovery of signaling pathways and transcription factors that link protein modification to changes in gene expression. The data

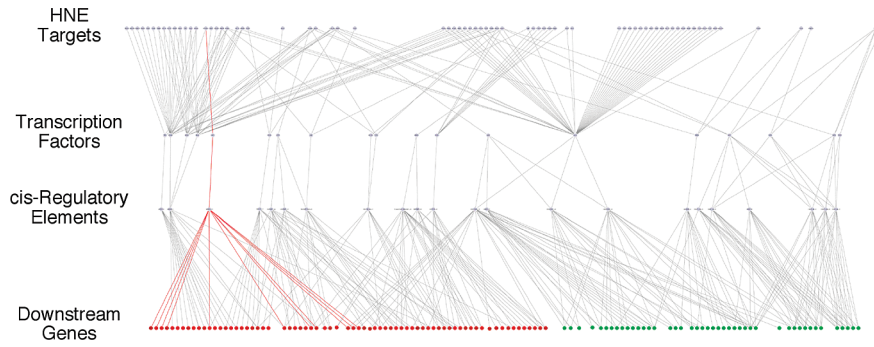

FIGURE 7. Network relating protein modification and gene expression changes induced by HNE. The dots at the bottom of the diagram represent genes that are upregulated (red) or downregulated (green) by HNE treatment. These genes are connected to the *cis* regulatory elements that control their expression, which are connected to the transcription factors that bind to them. Lines drawn from protein targets of HNE modification to the transcription factors controlling gene expression represent linkage by direct interaction or via signal transduction pathways that establish a plausible link between protein modification and altered transcriptional activity. The linkages representing control of the heat shock response are highlighted in red. This analysis was provided by Bing Zhang using data from refs 15 and 21.

summarized in Figure 7 illustrate the complexity of the chemistry and cellular responses to electrophile stress. It also identifies candidate transcription factors and pathways for study. To prioritize our studies of the relation between protein modification and cellular responses, we constructed expression vectors containing individual response elements upstream of the luciferase gene, which we transfected into RKO cells prior to treatment with HNE. This enabled a semiquantitative comparison of the magnitude of the transcriptional response induced from different signaling pathways following HNE treatment. The most robust response was observed with expression vectors under the control of the heat-shock response element. This provided a biological rationale for efforts to define the mechanism of activation of the heat-shock signaling pathway.

HNE and the Heat Shock Response

The inventory of HNE-modified proteins contained several heat shock proteins, including Hsp60, GRP78, Hsp70 (Hsp72), and Hsp90.[19] Likewise, microarray data revealed a dramatic increase in heat shock-regulated transcripts in HNE-treated RKO cells.[21] The inducible expression of heat shock genes caused by heat or chemical stress is principally controlled by the latent transcription factor, heat shock factor 1 (HSF1). A fundamental step in the activation of HSF1 is nuclear translocation.[24,25] In the absence of stress, HSF1 is retained in the cytoplasm by inhibitory associations with Hsp70, Hsp90, and various cochaperones.[26] We demonstrated that HNE promotes the nuclear translocation of HSF1 using Western blot analysis, and showed the enhanced transcription of a luciferase reporter gene under the regulation of a conserved heat shock element.[27]

We hypothesized that the process by which HNE enhances heat shock gene expression involves the modification of Hsp70, 90, and other chaperones, causing the release and nuclear translocation of HSF1. HNE treatment has been shown in vitro to adduct specific amino acid residues on both Hsp70 and Hsp90, which correlates with their reduced ability to bind and properly fold client proteins.[28,29] We performed coimmunoprecipitation experiments with myc-tagged Hsp70, demonstrating that its association with HSF1 is disrupted by HNE treatment. This occurs at HNE concentrations that affect Hsp70 modification, HSF1 nuclear translocation, luciferase expression from heterologous expression vectors, and heat shock gene expression. We are presently determining the sites of Hsp90 and Hsp70 modification and the functional consequences of HNE modification both in terms of HSF1 binding as well as its affiliation with other specific client proteins.

To assess the importance of the heat shock response in mediating the cellular response to electrophile stress, siRNA was used to silence HSF1, thereby attenuating heat shock gene expression in HNE-treated cells.[27] Control and HSF1-deficient cells were then exposed to HNE, and various responses measured including viability and gene expression changes. siRNA knockdown of HSF1 was nearly complete, and residual expression of the transcription factor was vanishingly small. Cells lacking HSF1 were profoundly more sensitive to the toxic effects of HNE than were cells that retained HSF1 (Figure 8). This suggests that HSF1-induced gene expression is an important protective response that is mounted by cells following electrophile treatment. In fact, in our studies, the cytoprotective role of HSF1 exceeds that of Nrf2 (the transcription factor responsible for the antioxidant response) based on a comparison of HNE toxicity and apoptotic markers in HSF1-

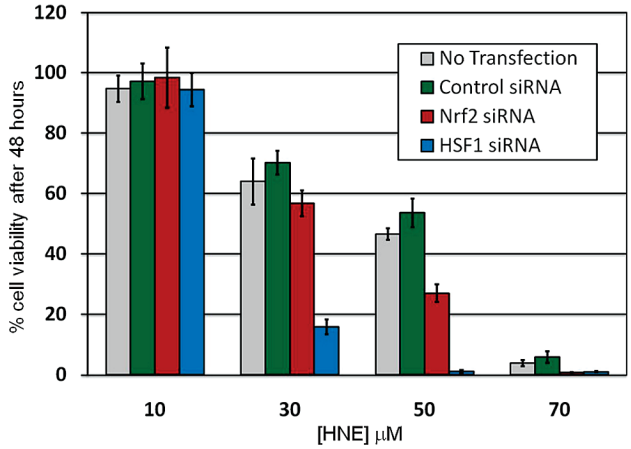

FIGURE 8. Comparative viabilities of HSF1- and Nrf2-silenced RKO cells treated with HNE.

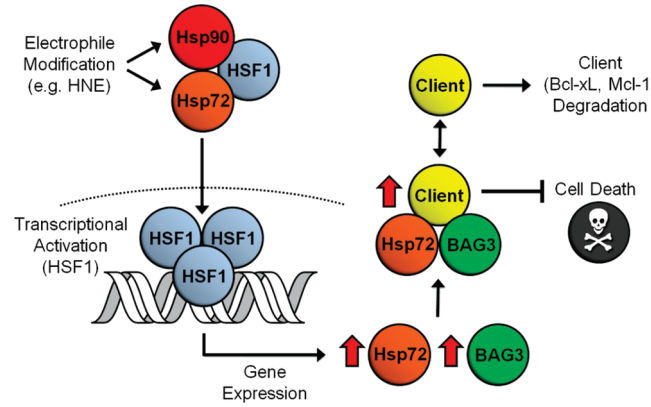

FIGURE 9. Proposed role for HSF1-enhanced BAG3 and Hsp72 expression in mitigating cell death based on increased levels of antiapoptotic client proteins.

silenced and Nrf2-silenced cells.[27] This observation implies that heat shock, at least in our cellular model, has greater significance than the antioxidant response in abating cell death caused by exposure to reactive electrophiles. It also illustrates the value of simultaneously monitoring multiple signaling pathways rather than a single one.

The mechanisms underlying the reduced viability of HSF1-deficient cells are varied, but the ultimate result is an increased sensitivity to HNE-induced apoptosis. HSF1-silenced cells showed dramatically elevated levels of JNK1 phosphorylation, which is a trigger for apoptosis, as well as decreased levels of Bcl-xL, which is an inhibitor of apoptosis. The reduction in Bcl-xL is due to diminished protein levels rather than reduced mRNA expression, which suggests that the inability of HSF1-silenced cells to mount a heat shock response leads to an increased turnover of Bcl-xL protein.

The molecular basis by which HSF1 attenuates cell death was examined in greater detail by microarray analysis, by comparing gene expression profiles between control and HSF1-silenced cells, following the addition of either vehicle (0.5% DMSO) or HNE.[30] Gene ontology (GO) analysis was performed in order to categorize HSF1-regulated genes by specific attributes (GO terms) defined by the GO Consortium.[31] GO terms fall under three broad categories, including "Cellular Component", "Biological Process", and "Molecular Function." For example, under the category of "Biological Process", we closely examined HSF1-dependent genes with either known or hypothetical antiapoptotic functions (Table 1). Although the expression of over 1000 transcripts showed some degree of dependence on HSF1, relatively few antiapoptotic transcripts (GO term: Negative Regulation of Apoptosis) were represented in the data; examples include CLU (clusterin); CRYAB (α,β-crystallin); HSPB1 (Hsp27), and BAG3 (Bcl-2-associated athanogene 3).[30] At the concentrations of HNE evaluated, cell death occurs through an apoptotic pathway dependent on protein synthesis and involving cytochrome *c* release, caspase activation, and PARP cleavage.[32] Bcl-2 inhibits HNE-induced apoptosis by preventing mitochondrial pore formation and the resulting efflux of cytochrome *c* and other proapoptotic factors. Since BAG3 was identified by others as a Bcl-2-interacting protein,[33] we hypothesized that BAG3 induction by HSF1 plays a critical role in mitigating cell death, possibly by enhancing the expression or prolonging the half-lives of Bcl-2, Bcl-xL, and related antiapoptotic proteins. (Figure 9).

BAG3 belongs to a family of protein cochaperones (BAG1−6) that regulate diverse cellular processes, including proliferation, migration, and apoptosis.[34] To evaluate the proposed role of BAG3 in facilitating cell survival, siRNA was used to silence its induction in HNE-treated cells. Knockdown of BAG3 enhanced HNE-induced cell death to the same extent as silencing HSF1, confirming the importance of BAG3 in the heat-shock-mediated defense against reactive electrophiles. We also observed that silencing BAG3 was associated with a dramatic loss in proteins belonging to the antiapoptotic Bcl-2 family, including Bcl-2, Bcl-xL, and Mcl-1. There was no reduction in the levels of mRNAs for any of the genes, suggesting that the reduction in their protein levels is due to enhanced protein turnover. Mcl-1 is of particular interest because it is one of the 10 most upregulated genes across all cancers and accounts for the resistance of many neoplasms to chemotherapy and radiation.[35] The mechanism we propose for the cytoprotective actions of BAG3 involves the stabilization of antiapoptotic Bcl-2 family members, perhaps by impeding their proteasomal or autophagic turnover.[30] Recent reports suggest that BAG3 is an important factor in carcinogenesis and tumor cell viability.[36−38] Growing recognition of a role for BAG3 in cancer suggests that its induction mediated by HNE

or other electrophiles derived from oxidative stress may facilitate or promote tumorigenesis. We are currently investigating the importance of BAG3 and other HSF1-regulated genes in mediating tumor cell viability and resistance to apoptosis.

Lessons from the HNE Case Study

The results of our combined studies of HNE-induced protein modification and gene expression reveal the complexity of cellular responses to electrophile stress. There are many proteins modified and many signaling pathways engaged. The ultimate cellular response reflects the input of multiple signaling networks and does not result from a key "molecular target" or dedicated signaling pathway. This contrasts with studies of cellular responses to electrophilic natural products (e.g., fumagillin) where specific molecular targets dictate the cellular response. A key difference between lipid electrophiles and natural products is the relative structural simplicity of the former. Molecules such as HNE contain electrophilic centers that are relatively unhindered and can, therefore, react with a variety of protein targets, and in some cases at multiple sites. In contrast, most natural products contain a high degree of structural complexity that limits access of the electrophilic center to relatively few proteins that possess complementary binding pockets. Although HNE reacts with a wide range of proteins, it and other simple electrophiles still modify only a small fraction of the total cellular proteome. Moreover, at concentrations that induce programmed cell death, many targets are modified at only one or two sites.[3] Thus, considerable selectivity in the reactivity of the proteome is observed even with such "nonspecific" electrophiles as HNE. It also appears that stability of the protein adduct is an important determinant of the ultimate cellular response to electrophiles: more stable adducts are associated with greater toxicity.[39]

Judging from our experience with HNE, cellular responses to electrophiles appear to be graduated based upon concentration and time of exposure. The gene expression changes induced by HNE treatment of RKO cells indicate that responses at low concentrations are mainly adaptive (e.g., heat shock response, antioxidant response) and are designed to protect against further damage. At higher concentrations, the adduct load on protein and DNA overwhelms these protective mechanisms and the cells undergo apoptosis. Finally, at extreme concentrations of electrophile, cells undergo a necrotic cell death. Despite the complex nature of the cellular response to electrophiles, the approach outlined in our case study proved effective in defining individual elements in the process of cellular adaptation. siRNA knockdown of key gene products (e.g., HSF1) coupled with detailed follow-up studies not only highlight the importance of the pathway of interest but can be used to decipher the mechanistic details of the overall cellular response.

Our decision to closely examine the heat shock response was motivated by two observations. First, we found that many heat shock genes were dramatically induced in HNE-treated RKO cells. Second, analysis of luciferase reporter constructs containing heat shock response elements confirmed that HNE promotes HSF1-dependent gene expression. Our hypothesis that HNE modification of Hsp70 or Hsp90 leads to the release and activation of HSF1 is supported by a reasonable body of in vitro and in vivo results.[19,27–29,40,41] However, it is also possible that modification of other cellular proteins leads to their unfolding, which attracts Hsp90 away from HSF1. Experiments are underway to test our hypothesis that modification of Hsp70 and Hsp90 is responsible for the HNE-induced heat shock response. A corollary of our hypothesis is that abundant cellular proteins such as Hsp70 and Hsp90 represent important targets that, when modified, can exert a profound cellular response. This is particularly important because, in addition to HSF1, Hsp90 has legion client proteins that control a wide variety of cellular functions. If the modification of Hsp90 indeed liberates client proteins, the potential for HNE and other relatively simple electrophiles to influence cellular function is great.

Our studies also highlight potential mechanisms by which tumor cells protect themselves from cytotoxic challenge presented by the innate immune system. Recent studies show that tumor cells can adapt to reactive oxidants and unfolded protein stress.[42] This adaptation manifests as a resistance to the cytotoxic insults of neutrophils and macrophages, plus a variety of chemotherapeutic agents. The heat shock response, mediated by HSF1, appears to be a major contributor to survival during such types of stress. This observation is substantiated by experiments performed in mice, where genetic deletion of HSF1 dramatically reduces the induction of skin tumors by the mutagenic and tumor-promoting combination of dimethylbenzanthracene and tetradecanoylphorbolacetate.[43] Further work must be performed to reveal the processes that make HSF1 both cytoprotective and protumorigenic. Our demonstration that BAG3 is essential in the HSF1-mediated resistance of RKO cells to electrophile-mediated cell death stress suggests BAG3 may be a feasible target to sensitize cancer cells to radiation, chemotherapy, and immune-mediated toxicities. However, additional genes are undoubtedly involved in HSF1-mediated resistance to electrophiles. We anticipate that some will be revealed through our ongoing global analysis of electrophile responses, and should contribute further to our understanding of cellular adaptations to stress.

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FOOTNOTES

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