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Histone adduction and its functional impact on epigenetics

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Abstract

Bioactive electrophiles generated from the oxidation of endogenous and exogenous compounds are a contributing factor in numerous disease states. Their toxicity is largely attributed to the covalent modification of cellular nucleophiles, including protein and DNA. With regard to protein modification, the side-chains of Cys, His, Lys, and Arg residues are critical targets. This results in the generation of undesired protein post-translational modifications (PTMs), that can trigger dire cellular consequences. Notably, histones are Lys- and Arg-rich proteins, providing a fertile source for adduction by both exogenous and endogenous electrophiles. The regulation of histone PTMs plays a critical role in the regulation of chromatin structure and thus gene expression. This perspective focuses on the role of electrophilic protein adduction within the context of chromatin and its potential consequences on cellular law and order.

Graphical abstract

Introduction

Oxidized lipids have been associated with a diverse range of pathological states, including atherosclerosis, cancer, diabetes, alcoholic and nonalcoholic liver disease, and neurodegenerative disorders.¹ Polyunsaturated fatty acids are particularly susceptible to

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Notes

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enzymatic and nonenzymatic oxidation by reactive oxygen species due to the presence of *bis*-allylic carbons between the double bonds (Figure 1).¹⁻² The products of nonenzymatic lipid peroxidation include various chain-length electrophilic species that are capable of covalently adducting cellular nucleophiles including lipids, DNA, and proteins.¹⁻² The generation of α,β -unsaturated aldehydes is of particular interest, as these electrophilic molecules contain two sites of reactivity and are thus capable of forming cyclic adducts, or protein-protein or protein-DNA cross-links.³⁻⁴ Enzymatic lipid peroxidation is catalyzed by lipoxygenases, cyclooxygenases (COX), and cytochrome P450s.² These controlled oxidations lead to the generation of a structurally diverse class of reactive metabolites, which include the electrophilic J-series prostaglandins (PGJ).⁵ An additional source of electrophilic molecules is peroxidation of DNA (Figure 2) via nonenzymatic reactions. In addition, irradiation of DNA may lead to the hydrolysis of the base, yielding reactive abasic DNA lesions. Akin to lipid electrophiles, these abasic sites are capable of forming stable adducts with nucleophilic biomolecules.

Electrophilic molecules are not solely generated through endogenous peroxidation mechanisms. Exogenous toxicants, such as environmental chemicals (e.g. vinyl chloride, benzene, diesel exhaust, cigarette smoke) and some drugs (e.g. acetaminophen, isoniazid), either are themselves or serve as precursors of bioreactive electrophiles.⁶⁻⁹ The toxicity of many environmental exposures has been linked directly to protein and/or DNA modification by electrophiles (i.e. adduction). For example, the cigarette smoke constituent acrolein (also a product of lipid peroxidation) has been identified as a major etiological agent of smoking-related lung cancer through its ability to covalently adduct DNA nonenzymatically.¹⁰ In addition, the toxicity of many exposures is mediated through the generation of electrophilic molecules via primary or secondary metabolism. An example of this is benzene, which is metabolized primarily to benzene oxide (via CYP2E1-mediated oxidation), and secondarily to benzoquinones (via NAD(P)H:quinone oxidoreductase 1). These metabolites are highly electrophilic and are associated with benzene toxicity.¹¹⁻¹² Regardless of the source, electrophilic biomolecules can wreak havoc on cellular homeostasis and have been identified as compounding factors in diseases associated with oxidative stress. While the generation and reactivity of electrophiles has been discussed in depth (see ^{1-2, 4}) the precise mechanisms by which they disrupt cellular law and order are less well defined.

As noted above, nucleophilic sites on both DNA and protein are susceptible to modification by electrophilic species. The adduction of DNA predominately occurs on the reactive nitrogen atoms of deoxyguanosine (dG) and deoxyadenosine (dA).¹³ Many of the resultant adducts are highly mutagenic, leading to base pair substitutions and frameshift mutations.¹⁴ While dG and dA are important targets for adduction, proteins are perhaps more heavily modified. The nucleophilic side chains of Cys, His, Lys, and Arg are all susceptible to modification.¹⁵⁻¹⁶ The thiolate anion of deprotonated Cys residues is the strongest of the protein-associated nucleophilic sites and usually reacts nonenzymatically with electrophiles via Michael-type additions that are readily reversible (Figure 3A).¹⁷⁻¹⁸ Although Lys residues are also prone to undergo a Michael-type addition following the interaction with an electrophile, the ϵ -amine group can undergo alternative nonenzymatic reactions, leading to more complex, stable products such as pyrroles and ketoamides that may persist in vivo (Figure 3).¹⁷⁻¹⁸ Further, the electrophilic levuglandins adduct Lys residues to generate stable

and immunogenic compounds (Figure 3). It should also be noted that both Lys and Arg can undergo oxidation themselves, generating reactive semialdehydes.¹⁹ Although this oxidation represents an interesting area for further research, the focus of this perspective is on the adduction and post-translational modification (PTM) of Lys residues, as these mediate a plethora of cellular responses.

PTMs often alter the activity, structure, and/or localization of their target protein. Although PTMs are reported on many amino acids, perhaps the most heavily modified residues are Lys and Arg.²⁰ The respective amine and guanidinium groups undergo numerous enzyme-mediated PTMs, including methylation (Lys and Arg), acetylation (Lys), citrullination (Arg), ubiquitination (Lys), and a plethora of acylations (Lys).²⁰ Methylation and acetylation are ubiquitously present across subcellular compartments; however, their role in the nucleus is vital to cell viability.²¹ Eukaryotic DNA is packaged in the nucleus via assembly into chromatin. The primary structural feature of chromatin is the nucleosome, which contains 147 bp of DNA wrapped around an octamer of histone proteins comprising two copies each of H2A, H2B, H3, and H4.²²⁻²³ The Lys- and Arg-rich tails of these proteins are heavily modified, leading to alterations in chromatin structure and/or recruitment of transcription factors, thus modulating gene expression.²² DNA is also subject modification. Occurring at the C-5 position of cytosine, DNA methylation is a heritable epigenetic mark that is largely associated with transcriptionally silenced regions of DNA.²⁴ This dynamic regulation of chromatin structure is tightly controlled through the enzymatic activity of PTM 'writers' (addition of a PTM) and 'erasers' (PTM removal) (Figure 4). Writers and erasers play a critical role in cellular homeostasis and are often implicated in disease pathogenesis.²⁵ As a result, these proteins are heavily targeted by the pharmaceutical industry for the treatment of numerous pathologies.²⁶ In addition to writers and erasers, histone PTMs are recognized by epigenetic reader domains, which are recruited to specific sites of modification. These domains are often present on writers and erasers and are typically required for proper enzymatic function. Reader domains have been identified for methylation marks on Lys and Arg, acetylated Lys, and phosphorylated Ser/Thr residues that are vital to cell homeostasis.²⁷ For example, recognition of acetylated Lys residues by bromodomains (BRDs) often results in the recruitment of transcription factors and enhanced gene expression^{26, 28-29}. BRD proteins are particularly important in mediating inflammatory responses through their ability to regulate NF κ B-induced gene expression.³⁰⁻³¹ This has garnered attention from a therapeutic standpoint, as inhibition of this interaction has been shown to be beneficial in rodent models of inflammation.³⁰⁻³³

This perspective focuses on the role of electrophilic protein adduction within the context of chromatin. Recently, our group evaluated the cellular targets for protein modification by the model lipid electrophiles 4-hydroxy-2-nonenal and 4-oxo-2-nonenal (4-HNE and 4-ONE, respectively). These two electrophiles, while structurally similar, displayed strikingly different adduction profiles (Figure 5). Of particular interest was the observation that histones, largely Cys-deficient proteins, were selectively targeted by 4-ONE, while non-histone, Cys-containing chromatin-associated proteins, were selectively targeted by 4-HNE. As highlighted by Zhu and Sayre, the previously reported 4-ONE adducts of Lys that were thought to result from Michael addition are, in fact, the much more stable 4-ketoamide.¹⁷⁻¹⁸ These adducts are structurally analogous to acylation products, which are typically observed

on histones (e.g. acetylation, crotonylation, succinylation).³⁴⁻³⁵ In addition to 4-ONE, histones have been identified as targets for adduction by additional electrophilic species, including levuglandins, acrolein, and formaldehyde.³⁶⁻³⁸ These modifications have been shown to disrupt nucleosome assembly and alter histone acetylation status.^{36, 39} This observation, and the Lys and Arg-rich content of histones, provides a fertile source of nucleophilic sites for adduction with potentially far reaching consequences on chromatin maintenance.

Electrophile sensing: cellular nodes for the regulation of gene expression

Traditional studies investigating the consequence of electrophile modification have utilized a targeted approach whereby the impact of modification on a single protein's function is elucidated. While these studies have provided invaluable mechanistic information on protein adduction, little is known about the mechanisms by which cells cope with this overt toxicity. Our laboratory has recently used a systems approach to relate protein targets of 4-HNE to gene expression changes.^{3, 40} By integrating protein adduction data sets with gene expression changes, protein-DNA interactions, and protein-protein interactions, it is possible to explore electrophile toxicity on a cellular scale.⁴⁰ This approach was not only successful in rediscovering known signaling pathways associated with electrophile stress (e.g. DNA damage) but was also used to generate novel hypotheses correlating protein adduction with cell toxicity pathways.⁴⁰ The integration of stress response signaling networks results in their convergence to regulate gene expression.

Histones are the most critical regulators of transcriptional processing through their recruitment of transcription factors via PTMs and their restructuring of chromatin to allow accessibility to DNA. Thus, electrophile-dependent modification of writers, readers, erasers, and histones themselves, presents a direct link between nonenzymatic protein adduction and gene expression changes. As the role of histone PTMs are relatively well-defined, we will first explore the potential deleterious impact of electrophile adduction to writers, readers, and erasers on histone function.

Adduction of epigenetic regulatory proteins

Writers

The post-translational modification of histones often alters chromatin structure and thus DNA accessibility.²² Histone acetyltransferases (HATs), which add an acetyl functionality to the ϵ -amino group of lysine residues, and methyltransferases, which methylate the ϵ -amino group of lysine or the guanidinium group of arginine, play a critical role in regulating gene expression.²⁵ It is conceivable to imagine that adduction of these enzymes via electrophilic species may prove deleterious to cell function and viability. This hypothesis was indeed tested by Ravindra et al. who demonstrated covalent adduction of the HAT, p300, by the electrophilic prostaglandin, 12 -PGJ₂.⁴¹ In this elegant study, the authors demonstrated modification of Cys1438, which resides in the substrate-binding pocket of p300. Adduction of p300 was found to markedly reduce its activity, leading to a decrease in acetylation of histone H3 at Lys 9 and 14 (H3K9 and H3K14, respectively).⁴¹ This was corroborated in the macrophage-like cell line, RAW264.7, where treatment with lipopolysaccharide to increase

¹²-PGJ₂ biosynthesis, resulted in depressed H3K9 and H3K14 acetylation. When cells were treated with indomethacin to prevent prostaglandin production by inhibition of COX-2, the lipopolysaccharide-mediated decrease in acetylation was reversed.⁴¹ In addition, Anavi et al. utilized HepG2 cells cultured in fatty acid-rich medium to investigate endogenous targets for lipid electrophiles.⁴² The 235 proteins identified as adducted in this study included three writer domain proteins, the N-lysine methyltransferase SETD8 and two HAT proteins, KAT6B and KAT8.⁴² The authors demonstrated site-specific 4-hydroxy-2-hexanal-(HHE)-dependent modification at His967 of KAT6B. Although mechanistic studies were not conducted to evaluate the potential deleterious impact of this modification on protein activity, collectively, these studies provide conclusive evidence that electrophilic species are capable of adducting writer domain proteins and potentially altering the epigenome.

DNA methylation is dynamically regulated through the activity of DNA methyltransferases (DNMTs).²⁴ Alterations in the activity and expression of these proteins have been associated with numerous disease states, further outlining their importance in genome maintenance.²⁴ While little work has been done to investigate the susceptibility of these proteins to electrophile adduction, the presence of an active site Cys suggests that they may be likely candidates. Liu et al. investigated the susceptibility of a DNMT1 analog (M. SsI) to modification by the anti-inflammatory compound, curcumin.²⁴ Although a dose-dependent decrease in DNMT1 activity was observed in the presence of curcumin, with an IC₅₀ of ~30 nM, the precise mechanism of action is unknown. Curcumin is an interesting compound, as its effects are believed to be through its oxidation, giving rise to secondary electrophilic intermediates that readily react with nucleophilic thiols.⁴³

Readers

As indicated, reader domains are often present on both writers and erasers.^{26, 44} As a result, it is difficult to discern studies evaluating the adduction of a particular reader domain without knowledge of the modification sites. An example of this complexity lies with the studies evaluating adduction of p300,⁴¹ a protein comprising eight domains. Of these, the BRD and plant homeodomain (PHD) are reader domains.⁴⁵ Although the site of ¹²-PGJ₂ adduction was found to lie in the HAT domain, it is highly conceivable that other domains within this protein may be susceptible to modification. This is a particularly intriguing notion when discussing PHD fingers, which bind to methylated Lys residues.²⁶ Although adduction of PHD fingers has yet to be reported, they contain a Cys₄-His-Cys₃ motif, which should be an attractive target for nonenzymatic electrophile adduction. If such adducts occur, they would likely compromise efficient targeting of the modified PHD domain to its correct epigenetic marks.

BRDs have become an extremely attractive target for therapeutic intervention in a growing number of diseases associated with inflammation.^{30, 32} These proteins bind to acetylated Lys residues on histone and non-histone proteins, most notably NFκB.⁴⁶ Acetylation of NFκB at Lys310 mediates inflammatory signaling through the recruitment of BRD4 and additional co-activators that stimulate the transcription of NFκB target genes.⁴⁷ In addition, BRDs were recently identified as key regulatory proteins in suppressing the expression of Nrf2-responsive genes through direct binding.⁴⁸ These data suggest a very attractive hypothesis

whereby nonenzymatic lipid electrophile modification of BRDs may inhibit their binding to Nrf2, thus releasing this transcription factor and allowing for activation of the antioxidant response. To date, BRDs have not been identified as targets for modification by electrophilic species; however further studies are clearly warranted.

Erasers

Although not directly related to chromatin regulation, studies of electrophile-dependent modification of eraser proteins in the mitochondria provide an interesting model system. PTMs play a critical role in mitochondria, where an estimated 20% of all proteins are acetylated.⁴⁹⁻⁵⁰ Perturbations in mitochondrial Lys acetylation have been linked to metabolic derangements, including alcoholic liver disease, where the levels of Lys acetylation are elevated up to 3.5-fold.^{49, 51} Alcoholic liver disease is an excellent model to study chronic oxidative stress and the role of lipid peroxidation and protein adduction in disease.⁵² The sirtuins are a class of deacylase enzymes, which includes three mitochondrial isoforms.⁵³ The role of these eraser proteins in modulating Lys acetylation under pathological conditions has been studied in depth.⁵⁴ Using a model for alcoholic liver disease, Fritz et al. were able to demonstrate adduction of the mitochondrial deacetylase, sirtuin 3 (Sirt3) by 4-HNE.⁵¹ Adduct levels were significantly elevated in mice consuming ethanol compared to control-fed counterparts. Further, using purified protein exposed to electrophile, MS revealed a Cys in the active site of the protein, Cys280, to be the most sensitive to modification.⁵¹ Adduction was found to drastically decrease the enzymatic activity of the protein, resulting in an increase in the acetylation status of its substrates. These data offer an interesting potential mechanism for the observed perturbation in mitochondrial Lys acetylation and suggest that electrophiles may mediate alterations in PTMs via a secondary mechanism. In addition, the nuclear-localized Sirtuin, Sirt1, has been identified as a target for nonenzymatic protein adduction in a murine model for aging-related ischemia.⁵⁵ Although a significant reduction in Sirt1 activity was observed, levels of Sirt1 target substrates were not investigated. Therefore, the precise impact of Sirt1 carbonylation in this model of ischemia remains open for interpretation.

Due to their major role in the regulation of gene expression and implications in disease, histone deacetylases (HDACs) have been heavily targeted by the pharmaceutical industry. To date, HDAC inhibition has been utilized as an effective therapeutic strategy for the treatment of cancers, diabetes-induced kidney injury, HIV, and cystic fibrosis.⁵⁶⁻⁵⁹ HDAC1, 2, and 3 contain conserved, surface-exposed Cys residues that are prime targets for lipid electrophiles.⁶⁰ These Cys residues have been shown to be modified by 4-HNE and the electrophilic prostaglandin, 15d-PGJ₂, both resulting in a dose-dependent decrease in deacetylase activity.⁶⁰ Treatment of cells with 15d-PGJ₂ resulted in an increase in H3 and H4 acetylation which facilitated an increase in antioxidant- and heat shock-responsive gene expression. This was found to be a direct result of adduction by 15d-PGJ₂ at Cys261 and Cys273 of HDAC1.⁶⁰ In addition, our own proteomic inventories have identified both HDAC1 and 2 as targets for modification by 4-HNE (Figure 5). Much akin to the sirtuins, adduction of HDAC enzymes may have far reaching consequences on the cellular responses to electrophilic stress by altering the acetylation status of histone proteins. Collectively, these studies propose the concept that electrophile susceptible proteins may initiate signaling

through ‘nodes’ that alter gene expression through secondary mechanisms. However, a much more direct hypothesis still exists – what happens when histones themselves are modified by electrophilic species? This concept offers an attractive avenue for research and remains the topic for the remainder of this perspective.

From histones to DNA: implications for adduction on epigenetics

Histones are adducted by electrophilic lipid peroxidation end products

Histone PTMs have been studied in depth for nearly two decades; however, most of this work has focused on modifications that are enzymatically regulated.²² The potential for nonenzymatic histone adduction remains an interesting notion, as such adducts are likely to be controlled by the laws of mass action. Indeed, 4-ONE adducts have been reported on histone Lys residues following the exogenous addition of electrophile to cultured cells.³⁹ Qualitative measurements of histone adduction using alkyne-tagged 4-ONE and click chemistry demonstrated H2B and H3 to be the most heavily modified histones in cells treated with electrophile.³⁹ These studies also evaluated site-specific modifications on histones using MS to reveal adducts on critical epigenetic sites, including H3K27. Further, adduction of this critically important modification site was detected in RAW264.7 macrophages stimulated with a lipopolysaccharide mimetic, suggesting a potential role for this PTM in mediating inflammatory responses. Although the precise role for 4-ONE-dependent histone modification in disease pathogenesis remains unclear, it was found to disrupt nucleosome assembly in *in vitro* analyses, suggesting potential deleterious effects on chromatin structure and integrity *in vivo*.³⁹

In addition to 4-ONE, additional products of arachidonic acid peroxidation have been found to nonenzymatically modify Lys residues on histones. Carrier et al. first identified levuglandin adducts on histone H4 in cultured RAW264.7 macrophages and the lung carcinoma cell line A549.³⁷ Adduct levels were significantly elevated following cytokine stimulation and were positively correlated with COX-2 expression, suggesting a potential role in mediating inflammatory responses. These levuglandin-histone adducts were also confirmed in rat liver tissue, again correlating with COX-2 expression. This *in vivo* identification of histone adducts has been further confirmed by Mont et al. who identified all four-core histones as targets for modification in whole lung homogenates using immunoprecipitation procedures with antibodies targeting the isolevuglandins.³⁸ Further, immunoblotting of chromatin fractions with this antibody demonstrated adduction of H3 and H4 in murine lung.

While endogenous lipid peroxidation constitutes a major source of electrophilic species in the cell, exogenous sources also have the potential to nonenzymatically adduct histones. In studies reported by Chen et al., the cigarette smoke constituent acrolein was found to adduct non-nucleosomal histones, with H2B being the most susceptible to modification.³⁶ The authors found that when cells were exposed to acrolein, the overall acetylation state of the four histones was significantly decreased on newly synthesized histones, thus altering nucleosome assembly, similar to observations reported by Galligan et al.^{36, 39} Due to the nature of these studies, where bolus administration of electrophile is applied to cell media, it is very likely that additional, indirect effects of acrolein exposure may be playing a part in

the observed decreases in histone acetylation (e.g. HAT adduction). Regardless of the mechanism, these data suggest that acrolein, and perhaps other electrophiles, may alter the underlying epigenetic state of the cell, potentially propagating stress responses (e.g. inflammation).

Modification of histones by DNA peroxidation products

The oxidation of the DNA backbone can lead to the generation of a plethora of reactive species.² One such product, base propenal, results from the oxidation of the C4' hydrogen of the deoxyribose ring, producing a highly electrophilic species that is placed in close proximity to the free amine groups of histone Lys residues.¹³ Indeed, base propenals have been demonstrated to nonenzymatically react with Lys residues, generating protein adducts analogous to those of malondialdehyde.⁶¹ Although base propenals have not yet been identified as a major source of histone modification, previous studies have demonstrated that the 3'-formylphosphate residue that is produced from the oxidation of the 5'-position of the deoxyribose is a major source for histone adducts.⁶² The formylphosphate moiety is readily transferred to the free amine of Lys residues, resulting in N-formylation.⁶² This modification is not only highly abundant (0.04-0.1% of histone Lys) but has a half-life on the order of cell turnover, suggesting that it may play a role in gene regulation. The sites of N-formylation on histones were identified by Wisniewski et al. who identified many of these modifications residing at epigenetic 'hotspots', including H3K18, H3K23, H4K12, and H4K31.⁶³ An additional source for N-formylation may arise as a by-product from lysine demethylation.⁶⁴ Although this has been shown to contribute minimally to the pool of N-formylation (<1%), with the vast majority arising from endogenous formaldehyde, it cannot be ruled out as a source for the adducts given their close proximity to histones.⁶⁵ Because this modification represents another acylation-type event, it is conceivable that N-formylation may alter nucleosome assembly and chromatin structure, much akin to 4-OH and acrolein modifications; however, follow-up studies using chromatin immunoprecipitation-sequencing (ChIP-Seq) are required to fully understand the biological role of this PTM.

An additional source for histone adduction results from the interaction of Lys residues with abasic DNA lesions (AP), notably C4'-oxidized abasic sites.⁶⁶ This mutagenic form of DNA damage is produced in excess of 10,000 lesions per cell each day, and Lys side chains play an active role in cleaving DNA at the site of this lesion via Schiff base formation.⁶⁷ In addition, 2-deoxyribonolactone, an alkali-labile DNA lesion, has been shown to interact with histone proteins resulting in DNA strand scission.⁶⁸ Histone Lys residues were found to increase the rate of strand scission up to ~43-fold compared to those in free solution, presumably through the electrophilic trapping of a butenolide intermediate.⁶⁸

The potential for histone-DNA crosslinks

The purpose of chromatin is to compact and regulate the estimated three billion base pairs in the human genome. As a result, proteins and DNA remain in extremely close proximity, greatly increasing the likelihood of DNA-protein crosslinks (DPCs). DPCs have been studied in depth; however, consistent with the scope of this perspective, we will focus on reports identifying and characterizing histone-DNA crosslinks.

1,3-Butadiene is a known carcinogen present in automobile exhaust and cigarette smoke.⁶⁹ Oxidation of 1,3-butadiene by cytochrome P450s yields 1,2,3,4-diepoxybutane, a potent crosslinking agent capable of generating both protein-protein crosslinks and DPCs.⁶⁹ Recently, Gherezghiher et al. conducted a proteomic screen to identify DPCs in human fibrosarcoma cells exposed to 1,3-butadiene. Among the 152 proteins identified, all four-core histones were found to contain these DPCs.⁶⁹ Indeed, Loecken et al. conducted an in depth investigation into the crosslinking nature of diepoxybutane in *E. coli* expressing human histone H2B.⁷⁰ Following exposure to electrophile, DPCs were found to contain histone H2B, although enhanced mutagenesis was not observed in this system. In addition to diepoxybutane, malondialdehyde has been shown to nonenzymatically generate histone-DNA crosslinks *in vitro*.⁷¹ In this case, the authors were able to demonstrate that crosslink formation proceeds through an initial reaction with the histone, followed by reaction with nucleobase. These crosslinks were also found to be extremely stable, with a half-life of ~13.4 days. Given that the half-life of a histone is typically on the order of ~1 day, this finding suggests that crosslinking impedes the ability of the cell to turnover these presumed damaged histones.⁷²

Although the potential for histone-DNA crosslinks exists, few studies have revealed such adducts. This may be due to multiple challenges hindering their detection. For one, histone-DNA crosslinks are likely a minor species compared to the relatively much higher levels of both histone and DNA adducts alone, and many may be unstable under physiological conditions. Thus far, exogenous administration of electrophile has been required to produce detectable quantities of histone-DNA crosslinks, suggesting that they may only form under conditions of significant toxicant exposure. In addition, current technology places limitations on our ability to detect and study DPCs via MS. This is largely a result of both the abundance of DPCs and the sensitivity of current MS instrumentation.

Detecting histone adducts: a problem of abundance and technology

Proteomic investigations into histones PTMs is a formidable task

Histones are Lys-rich and Cys-deficient proteins that are heavily modified through PTMs. The highly basic nature of these proteins, as well as the abundance of PTMs present, makes the analysis of histones by MS a significant challenge. This is due, in large part, to the difficulty in obtaining sufficient sequence coverage.⁷³ Due to the high number of Lys and Arg residues on histones, tryptic cleavage, as is typical in proteomic approaches, can often result in countless very small peptides, many of which are not retained on standard C₁₈ RP-HPLC columns. Furthermore, trypsin does not cleave at many Lys PTMs, so the products of tryptic digestion may vary widely between samples, depending on the level of modification present. To address these challenges, propionic anhydride can be used to selectively label unmodified Lys residues, thus resulting in a uniform “missed cleavage”. By propionylating all unmodified Lys residues, tryptic cleavage results in highly reproducible proteolysis only C-terminal to Arg residues. This increases the size of the tryptic peptides generated following hydrolysis, and therefore, their hydrophobicity and retention on C₁₈ RP-HPLC columns.⁷³ In addition, this strategy also results in the generation of more consistent tryptic peptides between samples, enabling direct comparison of differentially modified peptides

from the same site. Finally, the use of heavy isotope-labeled versus unlabeled propionic anhydride can provide the basis for relative quantification of peptides between samples. While approaches such as these are effective in modifying the pool of detectable peptides, they may also complicate discovery of novel modifications or modifications of unknown behavior following derivatization with propionic anhydride.

Due to the high degree of PTMs on histones, the heterogeneity of peptide masses in a given sample is exceptionally high, contributing to the complexity of proteomic approaches. Although acetylated and methylated Lys are reported to comprise the bulk of histone PTMs, additional PTMs such as Arg methylation, Ser/Thr phosphorylation, and Lys ubiquitination are widespread. In addition, many histone residues are reported to undergo numerous PTMs, drastically increasing the complexity of MS database searching.²⁰ For example, H3K27 may be acetylated, crotonylated, mono-, di-, or trimethylated, or ubiquitinated, depending on the epigenetic state of the cell²⁰. Proper selection of variable modifications is essential for accurate identification of peptides that may contain combinatorial PTMs. Insufficient interrogation of possible PTMs during database searching could result in decreased sequence coverage across peptides of interest. Thus, selecting the correct variable modifications is essential to obtaining sufficient MS coverage across the entire protein. It should be noted, however, that by simply increasing the number of variable modifications, the false-discovery rate concomitantly increases as well. This creates a “needle-in-a-haystack” scenario when attempting to identify and characterize low-level modifications resulting from electrophile adduction. Therefore, it is critical to manually interrogate raw spectral data to ensure correct PTM assignment. As a result, there are clear needs for improvement in both enrichment techniques and MS sensitivity to identify and quantify low-level histone PTMs.

Targeted approaches to identify histone adducts

Proteomic platforms have been used extensively to identify site-specific PTMs on histones.⁷⁴⁻⁷⁶ As with the study of any protein PTM, sample enrichment remains the major challenge that must first be addressed. This is particularly problematic when studying low-abundance protein adducts. One of the major advantages to studying histone modifications is the relative ease of obtaining high purity histone samples. As a result, one of three major targeted approaches is typically employed. Perhaps the most common strategy used to study histone PTMs relies on the solubility of histones in low pH solutions. Using 0.2N H₂SO₄, one can obtain highly enriched histone fractions, containing H1, H3, H4, H2A, and H2B (Figure 6).⁷⁷ The histones can then be separated based on their molecular weight using standard SDS-PAGE, and an in-gel tryptic digest of the separated protein precedes site mapping using MS. Conversely, in-solution tryptic digestions can be performed directly on crude histone preparations prior to elucidating site-specific modifications by MS analysis. In-gel and in-solution digests are often used as complementary approaches to obtain complete sequence coverage across the protein. While the H₂SO₄ method produces fractions containing predominately histones, the use of acid may result in the hydrolysis of relatively labile modifications. As an alternative, a high salt buffer (e.g. 420 mM NaCl) can be used to isolate chromatin-enriched samples from nearly any cell type or tissue.^{39, 77} Although non-histone chromatin proteins are present in these preparations, SDS-PAGE can again be used to selectively isolate the histone(s) of interest followed by in-gel digestion as described

above.³⁹ Perhaps the most critical step in the study of any PTM lies in the validation of the modification of interest. By interrogating the raw MS data, proper assignment of both y- and b-ions can be achieved. Improper assignments of protein PTMs has plagued this field and appropriate rigor must be applied.

Another common targeted strategy to study histone PTMs via MS relies on immunoprecipitation procedures, as are typically employed with other proteomic platforms. Using this approach, it is possible to isolate the histone of interest and perform either an in-solution tryptic digest or SDS-PAGE followed by in-gel digestion to identify the modification of interest. Although this technique has been utilized to successfully map numerous PTMs, it still relies on successful isolation of the histone of interest. Issues with antibody specificity have plagued this area of research, especially with regards to PTM-specific histone antibodies.⁷⁸ Conversely, immunoprecipitations can also be performed using antibodies targeting the electrophile of interest, rather than the protein. This is largely hindered by the lack of commercially available antibodies targeting electrophile modifications.

The application of chemical biology approaches to study protein adducts is becoming increasingly common.^{3, 39, 79} These approaches largely began with the development of carbonyl-reactive hydrazide probes.⁸⁰⁻⁸¹ Molecules such as biotin hydrazide have been utilized to selectively isolate aldehyde-adducted biomolecules in conjunction with streptavidin affinity. While these probes have yielded interesting results, they are relatively 'dirty' in that they react with oxidized glycosylation modifications as well.⁸² A marked improvement in affinity enrichment techniques was established with the introduction of click chemistry, which has enabled researchers to enrich proteins adducted by alkyne- or azide-tagged electrophiles (Figure 7). The use of a UV-cleavable biotin tag drastically increases sample enrichment and reduces non-specific protein targets, allowing for the isolation and identification of proteins susceptible to modification by the electrophile of interest.^{39, 74, 79} Further, the use of stable isotope tagging has allowed for the accurate site-specific assignment and quantitation of electrophile-sensitive peptides.⁷⁴⁻⁷⁵ This approach offers accurate quantitation of not just susceptible proteins, but precise marks on individual proteins. Traditional site-specific quantitation requires heavy-labeled peptides; however, this approach circumvents this problem by applying the heavy label to the affinity enrichment tag.⁷⁴⁻⁷⁵ In addition, by isolating only adducted peptides, the likelihood of site-specific assignment is markedly improved by decreasing the heterogeneity of the sample. Of course, the major caveat to this methodology is that it requires a 'clickable' tag on the electrophile of interest. For example, acrolein lacks an available C-C bond that can be modified with an alkyne, thus excluding the application of click chemistry. Nevertheless, much can still be done, and chemical biology approaches such as these will undoubtedly push the field forward at an accelerated pace. These targeted methods, while useful, all rely on a previous knowledge of the chemical structure of the electrophile; as a result, only information on that species can be deduced. As highlighted above, numerous electrophiles are generated during bouts of oxidative stress and thus, untargeted approaches are required to gain insight into unknown electrophilic modifications.

Untargeted approaches for the discovery of histone adducts

Researchers have developed a considerable interest in untargeted approaches for the discovery and assignment of unknown PTMs. The laboratory of Yingming Zhao has been at the forefront of this technology, using a relatively “unbiased” approach to identify novel histone PTMs including Lys 2-hydroxyisobutyrylation, butyrylation, propionylation, and crotonylation.^{34, 83-84} This approach utilizes tryptic digests of chromatin or histone preparations and high resolution MS/MS. The acquired data are then analyzed using PTMap, software developed to align sequences and enable detection of mass shifts resulting from PTMs.⁸⁵ The elemental composition of the mass shift can then be deduced to develop possible chemical structures. While this strategy has certainly resulted in major findings within the field of epigenetics, it may not be helpful when studying low-level modifications, such as those resulting from electrophile modifications. This likely comes back to the initial problem of enrichment, where other, more abundant PTMs are likely to suppress ions resulting from low-level protein adducts.

What is the biological significance of histone adduction?

The reports highlighted in this perspective have attempted to introduce a novel area for research in the field of epigenetics and toxicology. Although much work still needs to be accomplished, one can certainly ask what the biological significance of histone adduction might be. We offer four interesting hypotheses. Certainly the most obvious assumption is that histone adduction results in structural changes to the nucleosome core particle, and thus alters accessible regions of DNA. This hypothesis has been explored by our group and Chen et al. in studies that demonstrated a disruption in nucleosome assembly and structure following electrophile exposure.^{36, 39} Still, further work is required to evaluate how site-specific adducts (e.g. ketoamide adduction of H3K27) correlate with chromatin structure. A second hypothesis regarding the role of histone adducts is that steric hindrance blocks the correct placement of enzyme-mediated epigenetic marks, e.g. inhibition of acetylation. This concept has already been explored in the case of acrolein-dependent adduction that was found to decrease the overall acetylation status of histones.³⁶ It is likely that the nonenzymatic histone adducts blocked the availability for the modified site to undergo enzymatic addition of PTMs (e.g. acetylation or methylation). As highlighted, however, this observation should be taken with care, as it is possible that acrolein also be adducts HATs, thus leading to decreases in Lys acetylation.

Perhaps the most interesting hypothesis is that histone adduction itself is an epigenetic mark, akin to Lys acetylation. ChIP-Seq has been performed on most epigenetic marks in order to define their role in the regulation of gene expression. For example, H3K27 is often utilized as a marker for transcriptionally active (when acetylated) or transcriptionally silenced (trimethylated) regions of DNA.⁸⁶ To fully elucidate the potential role of histone adduction in transcriptional regulation, similar ChIP-Seq studies to provide information on the location of the adducts within the genome are required. It is highly conceivable that less compacted regions of the genome will be more prone to adduction, and thus no regions of enrichment would be observed. However, until these studies are performed, any assumptions regarding overall genome localization are pure speculation. It should be noted that ChIP-Seq can only be performed with covalent modifications, akin to the 4-ketoamide and lactam adducts and

that experiments such as these have not yet been accomplished in the case of nonenzymatic electrophile adducts, likely due to the relative lack of the necessary high fidelity adduct-specific antibodies.

Lastly, while most protein adducts are considered the result of “random” electrophile-nucleophile interactions, it is conceivable to imagine that adduction to histones is also regulated at the reader and eraser level. Reader domains that bind to adducted histones have yet to be elucidated; however, BRDs have been shown to possess a relative promiscuity for Lys acylation events, binding to Lys acetylation, crotonylation, and propionylation.⁸⁷ At the level of erasers, the growing number of modifications recognized as substrates by the sirtuins, ranging from Lys acetylation to the very bulky Lys glutarylation, suggests that these erasers may act on at least some electrophile adducts.⁸⁸ In a recent study by Jin et al., SIRT2 was found to remove 4-ketoamide adducts present at H3K23 and H3K27.⁸⁹ Further confirming this novel mechanism for the regulation of histone adducts, the authors demonstrated a marked increase in 4-ketoamide levels in SIRT2 knockout mouse embryonic fibroblasts exposed to lipopolysaccharide over wild-type controls.⁸⁹ Although each of these hypotheses generates its own level of excitement, the likely scenario is that the impact of electrophile adduction on histone structure and function is likely described best by an amalgam of all. Continuing studies will shed light on the role of electrophile-dependent histone modifications in health and disease.

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Biographies

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Lawrence J. Marnett received a B.S. in Chemistry from Rockhurst College in 1969 and a Ph.D. in Chemistry from Duke University in 1973 under the direction of Ned Porter. Following postdoctoral research with Bengt Samuelsson at Karolinska Institute and A. Paul Schaap at Wayne State University, he joined the faculty in Chemistry at Wayne State University in 1975. He moved to Vanderbilt University in 1989 where he is currently the Dean of Basic Sciences, Mary Geddes Stahlman Professor of Cancer Research, Professor of Biochemistry, Chemistry and Pharmacology and the Director of the A. B. Hancock Jr. Memorial Laboratory for Cancer Research at Vanderbilt University.

Abbreviations

COX	cyclooxygenase
PGJ	J-series prostaglandins
PTM	post-translational modification
BRD	bromodomains
4-HNE	4-hydroxy-2-nonenal
4-ONE	4-oxo-2-nonenal
HAT	histone acetyltransferases
DNMT	DNA methyltransferases
PHD	plant homeodomain
SIRT	sirtuins
HDAC	histone deacetylase
DPC	DNA-protein crosslink
ChIP-Seq	chromatin immunoprecipitation-sequencing

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Figure 1.
Peroxidation of polyunsaturated fatty acids and potential electrophilic products generated.

Figure 2.

A.) Generation of 3'-formylphosphate and base propenal through the peroxidation of DNA.

B.) Generation of abasic sites through irradiation of DNA. B = nucleobase; AP: abasic lesion; C4-AP: C4'-oxidized abasic lesion; L: 2-deoxyribonolactone.

Figure 3. Reactions typically observed through lipid electrophile adduction. 4-ONE is shown as a representative electrophile.

Figure 4.

A generalized scheme demonstrating the enzymatic activity of writers and erasers. Reader domain proteins bind to PTMs, often resulting in the recruitment of transcription machinery.

Figure 5.

A survey of proteins susceptible to modification by 4-HNE, 4-ONE, or both electrophiles. An investigation into chromatin-associated proteins reveals a relative specificity for 4-ONE to adduct Cys-deficient, Lys-rich proteins (i.e. histones). 4-HNE was found to adduct Cys-containing chromatin proteins, with a relative aversion to histones. Data was taken from ⁷⁹.

Figure 6.
Common approach for the targeted identification of histone modifications from biological samples.

Figure 7. Applications of click chemistry to identify adducted histones (left) or histone peptides (right) from cells treated with alkyne-tagged electrophiles. α 4-ONE is shown as a representative electrophile.