Stable Histone Adduction by 4-oxo-2-nonenal: A Potential Link Between Oxidative Stress and Epigenetics

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General Methods

Reagents. All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. 4-HNE, 4-ONE, alkynyl-4-HNE (a4-HNE) and alkynyl-4-ONE (a4ONE) were synthesized in the laboratory Dr. Ned Porter at Vanderbilt University. Kdo2-Lipid A (KLA) was purchased from Avanti Polar Lipids (Alabaster, AL). Arachidonic acid was purchased from NuChek Pre, Inc. (Elyssian, MN). Cell culture media was purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlas Biologicals ( Ft. Collins, CO). Purified histones and nucleosome assembly reagents were purchased from New England Biolabs (Ipswich, MA). SYBR Gold was purchased from Life Technologies (Grand Island, NY).

Cell culture. The colorectal cancer cell line, RKO, was cultured in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% FBS. Serum-free media (DMEM) was used for all treatments containing a4-HNE, a4-ONE or vehicle control (DMSO). RAW264.7 cells were cultured in DMEM and enriched with 25µM arachidonic acid for 24hrs. The media was removed and cells were washed once with DMEM. Cells were then stimulated via the addition of 100ng/µL KLA or vehicle control for 24hrs. Cells were washed and harvested in ice-cold PBS containing 5mM sodium butyrate. Cells were scrapped and pelleted via centrifugation at 1000 x g. All cell culture was maintained at 37°C under 5% CO₂.

Chromatin extraction. Methods were adapted from Torrente et al. and Shechter et al.1,2 Cell pellets were suspended in a hypotonic lysis buffer containing 10mM HEPES/KOH (pH 7.9), 1.5mM MgCl₂, 10mM KCl, 5mM sodium butyrate, supplemented with protease and phosphatase inhibitor cocktails. The suspension was placed on ice for 30 minutes to allow hypotonic swelling and nuclei were collected via centrifugation (1500 x g for 10 minutes at 4°C).2,3 Supernatants were discarded and chromatin was extracted from the pellet using a high salt buffer, consisting of 20mM HEPES/KOH (pH 7.9), 25% glycerol, 420mM KCl, 1.5mM MgCl₂, 0.2mM EDTA, 5mM butyrate, 20mM NaBH₄, and inhibitor cocktails. Pellets were sonicated on ice for 30 seconds and then rotated at 4°C for 2 hours. Chromatin was pelleted (1500 x g for 30 minutes at 4°C) and resuspended in a buffer containing 20mM HEPES/KOH (pH 7.9), 1.5mM MgCl₂, 0.2mM EDTA, 5mM butyrate, and inhibitor cocktails. Purity was validated via SDS-PAGE and coomassie blue staining.

Click chemistry. Methods were adapted from Ullery et al. 2011.4 Briefly, proteins were incubated with 1mM CuSO₄, 1mM TCEP, a biotin N₃-linker (0.2mM) and 0.1mM TBTA for a period of 2 hours in the dark at room temperature. Samples were then denatured in SDS loading buffer and resolved via SDS-PAGE to visualize modified proteins via streptavidin immunoblotting (Figure S2).

SDS-PAGE and immunoblotting. Samples were denatured in SDS loading buffer and heated at 95°C for 5 minutes. Proteins were resolved via SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked with Odyssey Blotting Buffer (Li-Cor Biosciences, Superior, NE) for 45 minutes at room temperature. Primary antibodies were incubated with membranes overnight at 4°C. Following 3x washes with TBS +0.1% Tween-20, infrared secondary antibodies (Li-Cor) were added in blocking buffer (1:5000) for 45 minutes. Blots were developed following 3 additional washes with TBST using the Odyssey Infrared Imaging System (Li-Cor).
In-gel digestion of histones. 2.5µg of chromatin was separated on a 15% SDS-PAGE gel and stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA). Four gel bands were excised corresponding to each histone based on molecular weight. Gel bands were cut into 1mm³ pieces, and treated with 45mM dithiothreitol for 30 minutes, followed by 100mM iodoacetamide for 45 minutes to alkylate all available Cys residues. After reduction and alkylation, gel pieces were destained with 50% MeCN in 25mM NH₄HCO₃. Four different digestion methods were employed to attain 100% sequence coverage over all four histones. For digestion of histones with trypsin, gel pieces were incubated with 10 ng/µL trypsin in 25mM NH₄HCO₃ for either 16 hours (Method 1) or 3 hours (Method 2) at 37 °C. For Methods 3 and 4, gel bands were first destained and then immersed in 5.9M propionic anhydride (prepared in methanol) and incubated for 20 minutes at room temperature. Propionylation was performed prior to reduction/alkylation and was repeated twice to ensure that all available lysine residues were propionylated. After each treatment with propionic anhydride, the solution was removed, gel pieces were dried under vacuum, and were subsequently rehydrated and rinsed three times with 100mM NH₄HCO₃. Digestion with trypsin was performed for either 16 hours (Method 3) or 1 hour (Method 4).

LC-MS/MS Analysis of Histone Modifications. Peptides were extracted following each method using gel dehydration (60% acetonitrile, 0.1% TFA); extracts were dried via speed vacuum centrifugation, and the peptides were reconstituted in 0.1% formic acid. Peptide mixtures were loaded onto a capillary reverse-phase analytical column (360µm o.d. x 100µm i.d.) using an Eksigent NanoLC Ultra HPLC and autosampler. The analytical column was packed with 20 cm of C18 reverse-phase material (Jupiter, 3µm beads, 300 Å or Aqua C18, 3µm beads, Phenomenex), directly into a laser-pulled emitter tip. Peptides were gradient-eluted at a flow rate of 500 nL/min, and the mobile phase solvents consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). A 90 min gradient was performed, consisting of the following: 0–15 min, 2% B (during sample loading); 15–60 min, 2–40% B; 60–70 min, 40–90% B; 70-73 min, 90% B; 73-76 min 90–2% B; and 76–90 min, 2% B (column re-equilibration). For select LC-MS/MS analyses, LC conditions were modified such that peptides were loaded at 0% B, and after sample loading, the peptide elution portion of the gradient consisted of 0-40% B from 15-60 min. Upon gradient elution, peptides were mass analyzed on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific), equipped with a nanoelectrospray ionization source. The instrument was operated using a data-dependent method with dynamic exclusion enabled. Full-scan (m/z 300–2000) spectra were acquired with the Orbitrap (resolution 60,000), and the top 16 most abundant ions in each MS scan were selected for fragmentation in the LTQ. An isolation width of 2 m/z, activation time of 10ms, and 35% normalized collision energy were used to generate MS2 spectra. Dynamic exclusion settings allowed for a repeat count of 2 within a repeat duration of 10 s, and the exclusion duration time was set to 15 s.

For identification of histone peptides, tandem mass spectra were searched with Sequest (Thermo Fisher Scientific) against a human subset database created from the UniprotKB protein database (www.uniprot.org). Due to the plethora of diverse histone modifications, multiple database searches were required for each sample to minimize false positives. These variable modifications included: +57.0214 (carbamidomethylation) on Cys; +15.9949 (oxidation) on Met; +14.0157 (monomethylation), +28.0313 (dimethylation), +42.0106 (acetylation) on Lys; +158.1306 (4-ONE Michael adduct) on Cys, His and Lys; +156.1150 (4-ketoamide), +138.1123 (Schiff-base 1), and +140.1279 (Schiff-base 2) on Lys. Additionally, for Methods 3 and 4, a variable mass of +56.0262 (propionylation) was applied to Lys. Search results were assembled
using Scaffold 3.0 (Proteome Software). Spectra of interest were inspected using Xcalibur 2.1 Qual Browser software (Thermo Scientific). Tandem mass spectra of all modified peptide precursors as well as spectra acquired of the corresponding unmodified peptide forms were examined by manual interrogation. The search parameters provided do not account for N-terminal processing and differences between trimethylation (+42.0471) and acetylation (+42.0106); therefore, all +42 mass shifts are identified as acetylation as manual validation is required for these peptides. Supplemental Table 2 contains a map for sequence coverage with the indicated method; however, only peptides containing 4-ONE modifications and gray shading were validated manually.

**In vitro assembly of nucleosomes.** Nucleosomes were assembled in vitro utilizing a previously developed salt dilution assay. Briefly, purified H2A/H2B dimers and H3/H4 tetramers were incubated with 208bp of DNA in a solution containing 2M NaCl. The salt concentration was diluted following 5, 30-minute incubations until the NaCl concentration reached 0.25M. To assess the impact of aldehyde modification on nucleosome assembly, assembled nucleosomes, H2A/H2B dimers, or H3/H4 tetramers were treated with increasing concentrations of either a4-ONE at 37°C for 60 minutes or 5mM acetic anhydride for 60 minutes on ice. Excess a4-ONE was quenched by the addition of 10mM N-α-Cys, whereas excess acetic anhydride was quenched with 10mM N-α-Lys. Aliquots of each sample were mixed with glycerol and resolved on a native-PAGE gel. The gel was stained in the dark with SYBR Gold for a period of 40 minutes and nucleosomes were visualized through excitation at 655nm. Acetylation was visualized via standard immunoblotting using antibodies directed against acetylated lysine (Cell Signaling, Danvers, MA). To ensure histones were present in nucleosomal bands, SYBR Gold-stained gels were then washed with ddH₂O and stained with Coomassie blue.

**References**

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Figure S1. Products of lysine adduction by 4-ONE.
Figure S2. (A) Cell treatments and isolation of chromatin were performed as described in Materials and Methods. (B) Overview of the click method utilized in these studies. Chromatin as isolated in (A) was clicked to biotin and histone adducts were selectively visualized using an IR-streptavidin and affinity. NaBH₄, sodium borohydride; TBTA, Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; TCEP, tris(2-carboxyethyl)phosphine; CuSO₄, copper sulfate.

A. Biotin

Histone

SDS-PAGE

IR-Streptavidin

B. a4-ONE

Histone

Nuclei Isolation
High Salt Buffer
50mM NaBH₄
Sonication

Chromatin

0.1mM TBTA
1mM TCEP
1mM CuSO₄
DMSO
Figure S3. (A) RKO cells were treated with increasing concentrations of either a4-HNE (left) or a4-ONE (right). Chromatin was extracted and adducted proteins were visualized using click chemistry and streptavidin visualization. Histones were selectively modified by a4-ONE while showing minimal reactivity with a4-HNE. H2B and H4 demonstrated the highest reactivity following changes in both concentration (A) and time (B). Coomassie brilliant blue (CBB) is shown as a loading control. Streptavidin blots are the same as depicted in Figure 1.

A.

| [aldehyde] | a4-HNE | a4-ONE |
|------------|--------|--------|
| -          | 5µM    | 10µM   |
|            | 25µM   | 50µM   |

WB: Streptavidin

B.

25µM aONE

| Min | 0    | 30   | 60   | 180  | 360  |
|-----|------|------|------|------|------|

WB: Streptavidin

CBB
Figure S4. Methods used for MS analysis of histones. Peptides identified with each method can be found in Supplemental Table S1.
Figures S5. MS/MS of the peptide containing a 4-ketoamide adduct on H3K23.
Figures S6. MS/MS of the peptide containing a 4-ketoamide adduct at H3K27.
Figures S7. MS/MS of the peptide containing a Michael adduct at H2BH82.
Figures S8. MS/MS of the peptide containing a Michael adduct at H2BH09.
Figures S9. MS/MS of the peptide containing a 4-ketoamide adduct at H2BK116.
Figures S10. MS/MS of the peptide containing a Michael adduct at H2AH123.
Figures S11. MS/MS of the peptide containing a 4-ketoamide adduct at H4K79.
Figure S12. *In vitro* assembly of nucleosomes as described in “General Methods”. These methods were utilized in Figures 4 and S12. Red indicates treatments corresponding to the gel presented in Figure 4.
Figure S13. Acetylated histones disrupt canonical nucleosome formation. (A) SYBR Gold staining of histones treated with AcAn disrupts nucleosome formation. (B) Immunoblot for AcLys demonstrating efficient acetylation of histones. (C) CBB of (A) AcAn, acetic anhydride.

Lanes: 1 – 100bp ladder
2 – DNA only
3 – DNA + AcAn
4 – Nucleosome
5 – Nucleosome + 100µM a4-ONE
6 – Nucleosome + AcAn
7 – Pretreated H2A/H2B, Pretreated H3/H4
8 – Pretreated H2A/H2B
9 – Pretreated H3/H4
Table S1. Composite list of the histone peptides identified using the methods described in Figure 2.

Table S2. A proteomic screen of chromatin reveals seven sites of modification by 4-ONE. Chromatin extracted from RKO cells treated with a bolus dose of 4-ONE (250µM) was subjected to SDS-PAGE and in-gel digestions as described in Figure 2; sites of modification were identified using LTQ Orbitrap Velos MS. Four adducts resulted from the stable addition of a 4-ketoamide adduct on a lysine residue. Sites of adduction are indicated as *; pr: propionylated lysine; species number corresponds to Figure S1.