Levuglandin Forms Adducts with Histone H4 in a Cyclooxygenase-2-Dependent Manner, Altering Its Interaction with DNA

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ABSTRACT: Inflammation and subsequent cyclooxygenase-2 (COX-2) activity has long been linked with the development of cancer, although little is known about any epigenetic effects of COX-2. A product of COX-2 activation, levuglandin (LG) quickly forms covalent bonds with nearby primary amines, such as those in lysine, which leads to LG-protein adducts. Here, we demonstrate that COX-2 activity causes LG-histone adducts in cultured cells and liver tissue, detectable through LC−MS, with the highest incidence in histone H4. Adduction is blocked by a γ-ketoaldehyde scavenger, which has no effect on COX-2 activity as measured by PGE2 production. Formation of the LG-histone adduct is associated with an increased histone solubility in NaCl, indicating destabilization of the nucleosome structure; this is also reversed with scavenger treatment. These data demonstrate that COX-2 activity can cause histone addition and loosening of the nucleosome complex, which could lead to altered transcription and contribute to carcinogenesis.

Cyclooxygenase-2 (COX-2) expression is associated with the development of many cancers, and the enzyme plays a key role in the progression of chronic gastrointestinal inflammation to cancer. Predictably, treatment with COX inhibitors decreases a person’s total risk of cancer. Prevention studies as well as animal models suggest that increased COX-2 activity is both an early event in carcinogenesis, which contributes to the cellular transformation to malignancy, as well as a sustained event in some colorectal and lung cancers that can be associated with metastasis and poorer clinical prognosis. As predicted by these data, inhibiting COX-2 activity with non-steroidal anti-inflammatory drugs (NSAIDS) or COX-2-specific inhibitors over time reduces a person’s total risk of colon, breast, lung, and prostate cancers.

Despite the promise of these drugs in cancer prevention, the gastrointestinal toxicity associated with long-term NSAID treatment and increased cardiovascular events associated with COX-2-specific inhibitors limit their clinical use. A better understanding of the specific downstream contributions of COX-2 to carcinogenesis could lead to new treatments that bypass these undesirable effects.

The product of COX-2, prostaglandin H₂ (PGH₂), is converted enzymatically into other prostaglandins, and indeed PGE₁ is a well-described promoter of carcinogenesis. However, depending on the animal model, deletion of microsomal PGE₂ synthase-1 can either prevent or accelerate tumorigenesis, indicating that the contribution of COX-2 to cancer, particularly to cellular transformation, is probably multifaceted.

Besides enzymatic conversion, PGH₂ also spontaneously rearranges in aqueous solution to form the highly reactive levuglandins LGE₂ and LGD₂. The γ-ketoaldehyde levuglandins (LGs) constitute about 20% of total PGH₂ rearrangement products. Newly formed LGs react almost immediately with free amino groups, such as those in lysine, which leads to stable covalent LG-protein adducts measurable by mass spectrometry (Figure 1) or protein–protein cross-links. Following COX-2 activity, LG adducts of protein form in cells and in tissues. Proteins rich in lysine are thus especially susceptible to addition, and due to the perinuclear localization of COX-2 and a PGH₂ half-life measured in minutes, we speculated that it would be possible for PGH₂ to cross the 

Received: December 17, 2013
Revised: March 28, 2014
Published: March 31, 2014

Figure 1. Structure of the LG-lysyl adduct and the fragment ions monitored in positive ion mode (+H).
nuclear envelope before rearranging to LGE₂, allowing formation of the LG adduct on the lysine-rich histones.

We have identified LG-histone adducts in multiple cancer cell lines as well as rat liver, with highest measurable amounts of adduct on the H4 histone. Adduct formation is dependent on COX-2 expression and activity, and we have found a small-molecule salicylamine derivative that scavenges LG to reduce adduct formation on histones without affecting COX-2 activity. Lysines, with their short side chain and positive charge, are critical for histone ionic interaction with DNA, and we find that this interaction is decreased with the introduction of a bulky negative charge from LG. Covalent histone modifications are a major method of controlling gene expression. Changes to lysyl modifications of histones are associated with human cancer. These findings link COX-2 induction with perturbation of normal DNA–histone interactions and provide a novel role for the enzyme in carcinogenesis.

**MATERIALS AND METHODS**

Materials. All reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Methanol and acetonitrile were from Fisher Scientific (Pittsburgh, PA) and were HPLC grade or higher. 14C-Arachidonic acid (AA) was obtained from PerkinElmer Life Sciences (Boston, MA). The following γ-ketoaldehydes scavenger molecules were synthesized by V. Amarnath as previously described: pentylpyridoxamine (PPM), 3-methoxysalicylamine (3-MoSA), and 5-ethylsalicylamine (EtSA).

Treatment of Cells. To stimulate COX-2 expression, A549 or RAW264.7 cells were treated overnight with 5 ng/mL IL-1β (A549) or 10 ng/mL IFNγ (RAW264.7) in serum-free medium. When indicated, cells were pretreated with indomethacin, aldehyde scavengers (glucosamine, 3-MoSA, PPM, or EtSA), or vehicle (ethanol for indomethacin, H2O for scavengers) for 45 min and then given 20 μM Arachidonic acid (AA) or DMSO vehicle for 1 h before lysing.

Histone Extraction. Cultured cells were lysed in hypotonic buffer (10 mM Tris/10 mM NaCl/3 mM MgCl₂) containing 1 mM pyridoxamine and 100 μM indomethacin to prevent the artificial formation of LGE₂ during the lytic process. After allowing the cells to swell on ice, membranes were disrupted by addition of Triton X-100 (0.5% final concentration) and vortexing. Nuclei were isolated by centrifugation for 10 min at 1000 × g, and the resulting pellet was washed with PBS. Histones were extracted in 0.4 N H₃SO₄, precipitated with trichloroacetic acid, and washed with acetone, following ref 23. With this method, histones are the predominant proteins and contaminating nuclear proteins are reduced (Figure 3A). Histones were resolubilized in dilute NaOH, and pH was neutralized with HCl. Protein concentration was determined using the method of Bradford. For tissue, a portion of frozen tissue containing 40 mM sodium citrate and 1% Triton X-100 with 3 mM Trolox and 100 μM indomethacin. The supernatant was separated from settled debris, and nuclei were pelleted at 500 × g and washed. Nuclear pellets were frequently transferred to fresh tubes to avoid contamination by floating lipid debris; any remaining was removed with a cotton swab. Histones were extracted as above. All centrifugation steps were carried out at 4 °C.

Sample Preparation and Mass Spectrometry. Histone samples were prepared for mass spectrometry by addition of ammonium bicarbonate to 5 mM final concentration before digestion to single amino acids by protease step-digestion as previously described. In the case of immunoblots, proteins were digested directly off the nitrocellulose through incubation of the nitrocellulose strip in 30 μg/mL Pronase in ammonium bicarbonate buffer and later addition of aminopeptidase. All samples were centrifuged at 2000 × g for 10 min after final digestion to remove precipitate, spiked with 0.2 ng of [13C]-lysyl-lactam internal standard, and purified on prepared tC18 cartridges (Waters Corp., Milford, MA). Samples on tC18 cartridges were washed with water and then 30% methanol before being eluted in 80% methanol and concentrated by evaporation. Samples were evaluated by electrospray ionization (ESI) LC–MS/MS on a ThermoFisher TSQ Quantum triple quadrupole mass spectrometer in positive ion mode and quantitated by isotopic dilution as previously described, with the exception of a reduced flow rate of 0.1 mL/min.

Measurement of PGE₂. A sample of cellular media was taken just prior to lysis and centrifuged to remove any cellular debris. For PGE₂ analysis, samples were spiked with 2 ng of [1H₄] PGE₂ as an internal standard. Prostaglandins were isolated and derivatized for analysis by GC–MS, operating in negative ion chemical ionization (NICI) mode and monitoring selected ions as previously described. For the [1H₄] PGE₂ internal standard, m/z = 528. To account for the deuterium-proton exchange at the position C12 of [1H₃] PGE₂, the summation of the signals obtained at m/z = 530, m/z = 531, and m/z = 532 was performed.

 Autoradiography. A549 or RAW264.7 cells were treated overnight to stimulate COX-2 expression and given 20 μM 14C-AA (116 μCi) for 1 h. Histones were isolated as described above and separated on 4–12% SDS-PAGE gels (Life Technologies), which were stained with Coomassie and exposed to film for 2 weeks.

Salt Extraction. A549 cells, 60–80% confluent, were stimulated and treated with AA ± 500 μM EtSA before cells were scraped in lysis media for nuclear isolation as above. After addition of Triton X-100 and vortexing, 1.5 mL of each sample was aliquoted into an eppendorf and centrifuged to separate nuclei. Pellets were washed with PBS, and all buffer was removed. Nuclear pellets were resuspended in extraction buffer containing 0.6, 0.9, 1.2, or 1.5 M NaCl (with 10 mM Tris, pH 7.5, 3 mM MgCl₂, 0.5% NP-40, and protease inhibitor cocktail) and incubated for 10 min on ice as in ref 24. Following this extraction period, the nuclei were centrifuged at 16,000 × g to obtain the soluble fraction. This was sonicated, denatured at 95 °C, and analyzed by SDS-PAGE and Western blotting, using Ponceau stain to visualize proteins and confirm equal loading and anti-H4 antibody (Abcam, Cambridge, MA).

Statistical Analyses. All data were analyzed using Prism software (GraphPad, La Jolla, CA). Data are expressed as means ± SE, and statistical significance was determined using one-way ANOVA followed by Tukey’s post-test or Dunn’s multiple comparisons post-test, when appropriate. A p value of <0.05 was considered significant.

**RESULTS**

Levuglandins Form Adducts on Histones in Cultured Cells and Whole Tissue. With mass spectrometry, we have identified LG-lysyl adducts on histones in RAW264.7 macrophages (Figure 2A) as well as A549 cultured lung epithelial cells (Figure 2C). COX-2 is upregulated in these cells upon cytokine stimulation, and addition of exogenous AA leads to formation of LG-histone adducts. Formation of these adducts is blocked with indomethacin, further indicating a COX-dependent
mechanism (Figure 2A and C). Very few LG-histone adducts are formed in these cell lines without addition of exogenous AA, and PGE2 analysis of cell media from each group indicates there is comparatively little endogenous AA mobilized following induction of COX-2 (Figure 2B and D). Although few adducts are formed at basal levels in our cell lines, we find the LG-lysyl adduct in rat liver histones (Figure 2E), where levels correlate with COX-2 expression, demonstrating COX-2-dependent adduct formation under physiological conditions.

**LG-Histone Adducts Are Restricted to Specific Histone Isoforms.** Using 0.4 N H₂SO₄ to extract histones results in a relatively pure preparation, with histones corresponding to known molecular weights (Figure 3A; ref 23). Incubation of ¹⁴C-AA with stimulated A549 or RAW264.7 cells led to a ¹⁴C-containing band in the histone preparation that corresponded with H4 and H3/H2B, despite the fact that other histones are represented in equal or greater quantity (Figure 3B). We treated stimulated RAW264.7 macrophages or A549 lung carcinoma cells with 20 μM AA for 1 h and directly digested and analyzed the SDS-PAGE bands as labeled in Figure 3A. The H4 band yielded a predominant peak corresponding with the internal LG-lysyl standard, while lower or no signal was seen in the H3/H2B band and no corresponding peaks were seen in other bands (Figure 3C and D, data not shown). Thus, there is consistent evidence for formation of an LG-lysyl lactam adduct on H4. The radiolabeled AA product adducted to H3/H2B probably includes structures in addition to the LG-lysine lactam.
results, from two separate cell lines, suggest that there is specificity in the reaction of LG with histones.

Scavenger 5-Ethylsalicylamine (EtSA) Reduces LG-Histone Adduct Formation without Affecting COX-2 Activity. We have previously characterized a number of small-molecule γ-ketoaldehyde scavengers that did not inhibit COX-2 and then further determined a subset that was free of cytotoxicity. From this subset, we then screened a number of these scavengers for the ability to reduce formation of LG-histone adducts. In RAW264.7 cells, only the scavenger 5-ethylsalicylamine (EtSA) was able to block adduct formation (Figure 4A). EtSA also inhibited LG-histone adduct formation in stimulated, AA-treated A549 cells, without affecting PGE₂ production at the highest concentration tested (Figure 4B and C).

![Figure 4. Scavenger EtSA blocks LG-lysyl adduct formation in RAW264.7 and A549 histones, without affecting COX-2 activity. (A) Scavengers were screened in RAW264.7 cells for the ability to decrease LG adduct formation on histones. Scavengers used were glucosamine (GA), 3-methoxysalicylamine (3-MoSA), pentylypyridoxamine (PPM), and 5-ethylsalicylamine (EtSA). Cells were stimulated to express COX-2, pretreated 45 min with 500 μM scavenger or vehicle (H₂O), and given 20 μM AA for 1 h before lysing and extraction of histones. Histone proteins were analyzed by LC–ESI/MS/MS for LG-lysyl lactam adduct, n = 2. (B) Stimulated A549 cells were pretreated with 30, 300, or 1000 μM EtSA prior to 1 h with 20 μM AA, and histones were analyzed for LG-lysyl adduct. *p < 0.05 by one-way ANOVA followed by Dunnnett’s multiple comparisons post-test (n = 3). (C). A549 cells were stimulated, pretreated for 45 min with 1000 μM EtSA or H₂O vehicle, and given 20 μM AA for 1 h. Media was analyzed by GC–MS for PGE₂ (n = 3). There was no effect on PGE₂ production at lower doses of EtSA (data not shown).

![Figure 5. LG-lysyl adduct formation on histone H4 decreases DNA–histone interaction. A549 cells were stimulated and given DMSO vehicle (C lanes) or 20 μM AA for 1 h (A lanes). A subgroup of cells was treated with 500 μM EtSA 45 min prior to adding AA (E lanes). Nuclei were extracted with 0.6, 0.9, or 1.2 M NaCl buffer, and the supernatant was evaluated by Western blotting for histone H4. Shown is a representative Western blot (A) as well as the pooled results of 4 experiments (B). Different exposure times may have been used for the 0.9 and 1.2 M bands. ***p < 0.001 by one-way ANOVA followed by Tukey’s multiple comparisons post-test. NS, not significant.](image)

**DISCUSSION**

This study, establishing that COX-2 catalysis can cause changes in DNA–histone interactions through formation of LG-histone adducts, suggests a new hypothesis for the contribution of COX-2 to the etiology of cancer. Oxidative damage is known to cause N⁶-formylation of H1 histone, and epigenetic modification affecting COX-2 transcription is well-described, but the LG-lysyl histone adduct we describe here is an entirely novel finding that links inflammation and COX-2 activation with histone modification.

Here we demonstrate COX-2-dependent formation of LG-histone adducts and total cellular adduct levels of LG-lysine adducts without affecting PGE₂ production. These scavengers are orally bioavailable and able to decrease total LG-protein adduct levels when given to mice in drinking water. In future studies, aside from allowing investigation of LG-protein modification independent of COX activity, use of these scavengers may bypass the cardiovascular and gastric side effects seen with COX inhibitors.

Interestingly, not all histones are targeted, but cellular LG adducts seem to preferentially form on the H4 and, to a lesser extent, on H3/H2B. Whether this specificity is a reflection of
histone availability in the nucleosome, accessibility of lysine residues, or a more favorable microenvironment for adduct formation remains to be shown. Incubation with \(^{14}\)C-AA in cells led to a stronger autoradiographic band at H3/H2B compared to H4, while LC–MS analysis indicated that H4 was the major form adducted by LGs. This discrepancy can be explained by several mechanisms. Protein-associated radioactivity would come from any product derived from \(^{14}\)C-AA, explained by several mechanisms. Protein-associated radioactivity would come from any product derived from \(^{14}\)C-AA, explained by several mechanisms. Protein-associated radioactivity would come from any product derived from \(^{14}\)C-AA, explained by several mechanisms. Protein-associated radioactivity would come from any product derived from \(^{14}\)C-AA, explained by several mechanisms.

The complex patterns of lysyl acetylation and methylation comprise a "histone code" that regulates chromatin access and transcription; it is plausible that irreversible adduction of lysyl residues could disrupt this code or directly alter the access of DNA-interacting proteins. Changes in histone modifications are known to result in altered DNA methylation, deregulation of oncogenes, genomic instability, impaired DNA repair, and defects in cell cycle checkpoints. Changes in lysyl modifications of H4 in particular are a common hallmark of human cancers and are associated with a global loss of DNA methylation. Further elucidation of the effects of LG-histone adduction on histone modification, DNA-histone interactions, and transcription should increase our understanding of the molecular mechanisms whereby COX-2 contributes to cancer development and progression.

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**Author Contributions**

**Funding**

Supported by the Center for Molecular Toxicology, NIH P30 ES000267, American Cancer Society PF-09-127-01-DMC (E.J.C.), GM15431, and the Thomas F. Frist, Sr. Chair in Medicine (J.A.O.).

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The authors are grateful for the valuable advice of Scott Hiebert.

**ABBREVIATIONS**

LG, leovuglandin; COX-2, cyclooxygenase-2; NSAID, non-steroidal anti-inflammatory drug; PGH\(_2\), prostaglandin H\(_2\); PPM, pentyloxyridoxamine; 3-MOSA, 3-methoxysalicylamine; EtSA, 5-ethylsalicylamine; AA, arachidonic acid; ESI, electrospray ionization; NICI, negative ion chemical ionization

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