Title

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Journal

Chemical Research in Toxicology, 26(10)

ISSN

0893-228X

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Publication Date

2013-10-21

DOI

10.1021/tx400320u

Peer reviewed

Chemical Research in Toxicology

Rapid Report

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Dosimetry of N^6-Formyllysine Adducts Following [$^{13}C^2H_2$]-Formaldehyde Exposures in Rats

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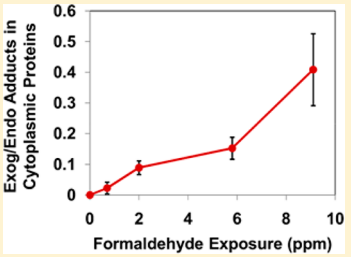
Supporting Information

ABSTRACT: With formaldehyde as the major source of endogenous N^6-formyllysine protein adducts, we quantified endogenous and exogenous N^6-formyllysine in the nasal epithelium of rats exposed by inhalation to 0.7, 2, 5.8, and 9.1 ppm [$^{13}C^2H_2$]-formaldehyde using liquid chromatography-coupled tandem mass spectrometry. Exogenous N^6-formyllysine was detected in the nasal epithelium, with concentration-dependent formation in total as well as fractionated (cytoplasmic, membrane, nuclear) proteins, but was not detected in the lung, liver, or bone marrow. Endogenous adducts dominated at all exposure conditions, with a 6 h 9.1 ppm formaldehyde exposure resulting in one-third of the total load of N^6-formyllysine being derived from exogenous sources. The results parallel previous studies of formaldehyde-induced DNA adducts.



Formaldehyde (FA) is classified as a known human carcinogen by IARC[1,2] and causes squamous cell carcinoma in rats.[2] There is sufficient epidemiological evidence for causing nasopharyngeal cancer but limited evidence for human leukemia.[1−3] Exposure to FA occurs from endogenous cellular processes, as well as environmental and occupational sources,[2,3] with plasma concentrations ranging from 13 to 97 μM.[3] As a reactive aldehyde, the toxicity of FA likely involves facile reactions with nucleophilic sites in molecules, including the formation of N^2-hydroxymethyl-dG (HM-dG) adducts in DNA, and DNA−protein and DNA−DNA cross-links, as well as Schiff bases with lysines in proteins.[2] We recently discovered that FA is a major source of N^6-formyllysine (FLys) in proteins,[4] along with oxidative and nitrosative stresses of inflammation.[5,6] Our data showed a concentration-dependent formation of FLys in cells exposed to [$^{13}C^2H_2$]-FA, while endogenous levels of FLys did not change during the exposure.[4] Here, we extend our previous cell and DNA adduct[7] studies to measure FA-induced lysine N^6-formylation in rats exposed by inhalation, using [$^{13}C^2H_2$]-FA to differentiate endogenous from exogenous adducts.

Fischer rats (6 w old, male, n = 3) were exposed to FA vapor by nose-only inhalation exposure of [$^{13}C^2H_2$]-FA for 6 h to produce final target exposure concentrations of 0 (air control), 0.7, 2, 5.8, and 9.1 ppm [$^{13}C^2H_2$]-FA. Rats were euthanized using an intraperitoneal barbiturate injection and tissues collected (Supporting Information). Protein extraction and amino acid quantification were performed as described previously.[4] Total, as well as cytoplasmic, membrane, and nuclear proteins were extracted from ∼10 mg tissue samples and the protein digested to amino acids using *Streptomyces griseus* protease. Lysine and FLys were quantified by liquid chromatography-coupled tandem quadrupole mass spectrometry (LC-MS/MS) with limits of detection of 10 and 1 fmol, respectively (the detailed protocol is in Supporting Information).

Endogenous (m/z 175→112) and exogenous (m/z 177→114) FLys along with the 4,4,5,5-[^{2}H]-FLys internal standard (m/z 179 →116) were monitored by LC-MS/MS (Figure 1). There were similar levels of endogenous adducts among different tissue types, with a range of 2−4 FLys per 10^4 lysines (Table S1, Supporting Information). Each tissue had comparable endogenous adducts in the control rats compared to rats exposed to the highest dose of 9.1 ppm [$^{13}C^2H_2$]-FA (Table S1, Supporting Information), which indicates that exposure to inhaled FA did not affect the endogenous adducts. However, exogenously derived FLys was only detected in proteins extracted from the nasal epithelium and not in distant tissues of the lung, liver, or bone marrow (Table S2, Supporting Information). In all samples analyzed from distant tissues, the exogenous adducts did not increase beyond the natural isotope abundance level of ∼0.7% for [M + 2] ion of FLys. In addition to total protein, the analysis of protein in cytosolic, membrane and nuclear compartments revealed exposure-dependent formation of exogenous FLys only the in nasal epithelium (Table 1 and Figure 2). The limited distribution of FLys to the

Received: September 3, 2013
Published: October 2, 2013

   dx.doi.org/10.1021/tx400320u | *Chem. Res. Toxicol.* 2013, 26, 1421−1423

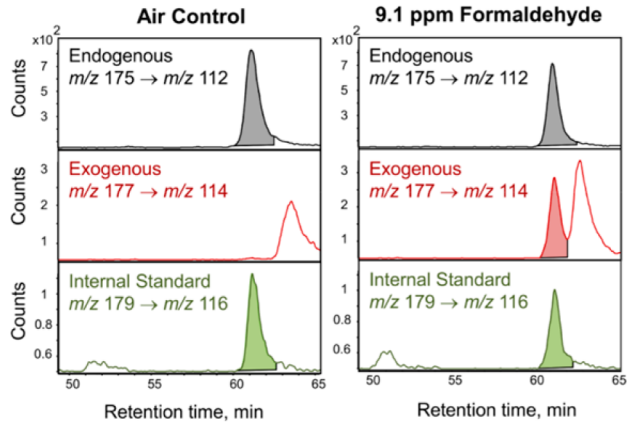


Figure 1. Inhalation of [$^{13}C^2H_2$]-FA distinguishes exogenous from endogenous sources of FLys in rats. LC-MS/MS signals for the three isotopomeric species, in cytoplasmic proteins extracted from the nasal epithelium.

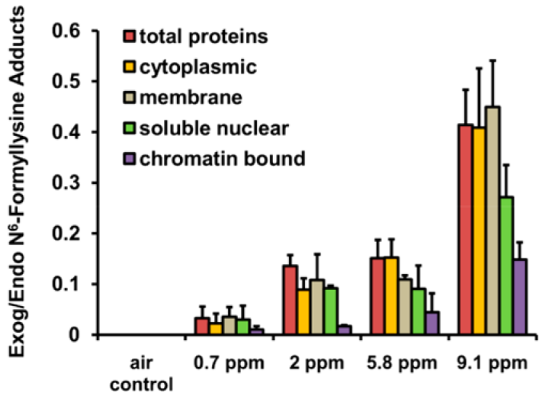


Figure 2. [$^{13}C^2H_2$]-FA causes a dose-dependent increase in exogenous FLys. Ratios of exogenous vs endogenous FLys in the nasal epithelium of rats exposed by inhalation to [$^{13}C^2H_2$]-FA for 6 h. Data represent the mean ± SD for n = 3.

nasal epithelium is consistent with our studies of FA-induced HM-dG formation[7] and suggests that inhaled FA is consumed in the nasal passages before it can be distributed to distant tissues.

The data also revealed that, at all doses, endogenous adducts dominated. There was a clear exposure−response relationship for lysine N^6-formylation across the range of inhaled FA doses (Figure 2), with exogenous adducts in total protein rising from <3% of endogenous adducts to >40% for a ∼10-fold increase in FA exposure (0.7 to 9.1 ppm). As shown in Table 1 and Figure 2, there was a lower amount of adduct formation in nuclear proteins compared to proteins from other cellular compartments. For example, a 9.1 ppm FA exposure produced 0.2 exogenous FLys adducts per 10^4 lysines in chromatin bound proteins compared to 0.8 and 0.7 residues in cytoplasmic and membrane fractions, respectively ($p < 0.05$).

These results point to several important features of FLys formation and FA toxicity. FLys has been shown to arise globally in proteins from different cell compartments as well as plasma proteins.[4] These observations, together with previous *in vitro* FA studies[4] and the relatively high FA exposures from environmental and endogenous sources,[2,3] point to FA as a major source of FLys in cells. Interestingly, endogenous levels of FLys were unaffected, even at the highest FA dose, which suggests that inhaled FA does not alter cellular FA production. The observation that background FLys levels are similar in proteins from all cell compartments suggests that the sources of this protein modification are balanced in the various compartments, consistent with the cellular abundance of FA due to the metabolism of xenobiotics and endogenous sources.[2,3] The exposure-dependent formation of exogenous [$^{13}C^2H$]-FLys in all compartments, with lower concentrations in the nucleus, is consistent with exogenous FA being consumed before entering the nucleus.

To further correlate protein and DNA adducts, exogenous/endogenous ratios of FLys in histone proteins (major proteins in chromatin) were plotted against the published values of HM-dG adducts[7] (Figure S1, Supporting Information), revealing FA-dependent increases for both [$^{13}C^2H_2$]-adducts, with ∼15-fold and 3-fold increases in protein and DNA adducts with exposures ranging from 2 to 9.1 ppm and from 5.8 to 9.1 ppm, respectively. The relative exogenous/endogenous ratio of DNA adducts was higher compared to histone adducts for the same FA dose (Figure S1, Supporting Information). For instance, at 9.1 ppm FA, the HM-dG adduct ratio was more than 3-times that of FLys (∼0.6 vs <0.2). Absolute amounts of FLys were always greater (FLys per 10^4 lysine vs HM-dG per 10^7 dG[7]).

The analysis of FLys sheds light on mechanisms of FA toxicity. Data from protein adducts complements previous studies of FA-induced DNA adducts in rats,[7] with our results showing strong correlations between protein and DNA adduct formation. Our results show that, similar to [$^{13}C^2H_2$]-HM-dG adducts, the exogenously derived FLys was only detected in nasal epithelium and not in distant tissues, with an exposure-dependent formation of exogenous adducts in total proteins as well as proteins in cell compartments (Figure 2 and Table 1). Moreover, both [$^{13}C^2H_2$]-HM-dG and [$^{13}C^2H_2$]-FLys follow similar patterns as a response to FA exposure (Figure S1, Supporting Information), even though the relative exogenous/endogenous ratios of HM-dG were significantly higher than those for histone adducts at the same FA dose. The difference

Table 1. N^6-Formyllysine Protein Adducts in Nasal Epithelium from Rats Exposed to [$^{13}C^2H_2$]-Formaldehyde

exposure	air control		0.7 ppm		2 ppm		5.8 ppm		9.1 ppm	
adduct type	endo[a]	exog	endo	exog	endo	exog	endo	exog	endo	exog
total protein	1.6 ± 0.1[b]	N.D.[c]	1.7 ± 0.1	0.06 ± 0.04	1.7 ± 0.2	0.23 ± 0.02	2.2 ± 0.4	0.33 ± 0.04	2.1 ± 0.1	0.86 ± 0.11
cytoplasmic	2.0 ± 0.4	N.D.	2.4 ± 0.3	0.05 ± 0.04	2.6 ± 0.2	0.23 ± 0.07	2.3 ± 0.8	0.35 ± 0.18	2.1 ± 0.4	0.84 ± 0.14
membrane	2.7 ± 0.8	N.D.	1.7 ± 0.3	0.06 ± 0.02	2.3 ± 0.7	0.23 ± 0.03	3.0 ± 0.2	0.33 ± 0.02	1.6 ± 0.3	0.74 ± 0.24
soluble nuclear	1.8 ± 0.3	N.D.	1.6 ± 0.3	0.05 ± 0.05	2.0 ± 1.0	0.19 ± 0.13	4.4 ± 0.3	0.39 ± 0.20	2.0 ± 1.0	0.53 ± 0.21
chromatin bound	1.7 ± 0.1	N.D.	1.6 ± 0.4	0.02 ± 0.02	2.4 ± 0.8	0.03 ± 0.01	2.1 ± 0.1	0.07 ± 0.05	1.5 ± 0.4	0.22 ± 0.01

[a]Endogenous (Endo) and exogenous (Exog) FLys for each FA exposure. [b]Data are FLys per 10^4 lysines and represent the mean ± SD for 3 rats. [c]N.D., not detected beyond the natural isotope abundance of ∼0.7% for the [M+2] ion of FLys (limit of detection of 1 fmol).

could be due to factors such as DNA guanine content compared to histone lysine content, different kinetics of formation, as well as target accessibility.

There have been many studies on the mechanisms of formaldehyde toxicity and carcinogenicity.[2] For instance, several found a nonlinear exposure-dependent formation of DNA damage in rats and nonhuman primates exposed to inhaled FA, with other studies showing that long-term FA exposures >6 ppm substantially increase squamous cell carcinoma in rats.[2] On the path to understanding the biological impact of FA, our results shed light on another pathway: formation of N^6-formyllysin in proteins, including histones. FLys has been mapped on conserved lysine acetylation and methylation sites in histones.[8,9] This observation, along with the chemical similarity of lysine N^6-formylation and N^6-acetylation, as well as our results showing FLys is refractory to removal by histone deacetylases,[4] suggests that FLys could interfere with the epigenetic function of histone modifications.[10] FLys from environmental and occupational FA exposure could thus contribute to FA toxicity and carcinogenicity.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and quantitative protein and DNA adduct data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This project was supported by the MIT David H. Koch Cancer Research Fund and the NIH/NIEHS (grants ES016450, ES005948, ES010126, and ESoo2109), NIH/NCI (grants CA026731 and CA103146), and the Texas Commission for Environmental Quality. LC-MS studies were performed in the NIEHS-supported MIT Center for Environmental Health Sciences. Funding for the FA exposures was provided by the Research Foundation for Health and Environmental Effects, a 501 (c)(3) organization.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

FA, formaldehyde; HM-dG, N^2-hydroxymethyl-dG; FLys, N^6-formyllysine; LC-MS/MS, liquid chromatography-coupled tandem quadrupole mass spectrometry

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