Endogenous Lipid Hydroperoxide-mediated DNA-adduct Formation in Min Mice**

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Despite intensive research over the last two decades, there are still no specific markers of endogenous lipid hydroperoxide-mediated DNA damage. We recently demonstrated that heptanone-etheno-2′-deoxyguanosine adducts are formed in the DNA of rat intestinal epithelial cells that stably express cyclooxygenase-2. Heptanone-etheno adducts can only arise from the reaction of lipid hydroperoxide-derived 4-oxo-2(E)-nonenal with DNA. This raised the possibility that similar adducts would be formed in vivo in settings where cyclooxygenase-2 expression is increased. Therefore, DNA-adduct formation was studied in C57BL/6JAPC\textsuperscript{min} mice, a colorectal cancer mouse model in which cyclooxygenase-2 is up-regulated. 15(S)-Hydroperoxy-5,8,11,13E-eicosatetraenoic acid is the major lipid hydroperoxide produced endogenously by cyclooxygenase-2. It undergoes homolytic decomposition to the DNA-reactive bifunctional electrophile 4-oxo-2(E)-nonenal, which forms heptanone-etheno adducts with DNA. A quantitative comparison was made of the heptanone-etheno-DNA adducts present in C57BL/6J and C57BL/6JAPC\textsuperscript{min} mice. Using highly specific and sensitive methodology based on stable isotope dilution liquid chromatography/tandem mass spectrometry, we have detected the endogenous formation of heptanone-etheno adducts in mamalian tissue DNA for the first time. In addition, we found that there were statistically significant increased levels of the heptanone-etheno-2′-deoxyguanosine and heptanone-etheno-2′-deoxycytidine adducts in the C57BL/6JAPC\textsuperscript{min} mice when compared with the control C57BL/6J mice.

Colorectal cancer is the second leading cause of cancer-related deaths in the United States. Each year ~130,000 people are diagnosed with colorectal cancer, and ~56,000 will die from the disease. Multiple large epidemiological studies showed that regular use of NSAIDs\textsuperscript{2} was associated with a reduction in the risk of cancer (1, 2). Furthermore, for individuals with inherited familial adenomatous polyposis, NSAID intake was associated with a reduction in polyp number and size (1, 3, 4).

Likewise, NSAIDs have demonstrated their efficacy to inhibit carcinoma formation in animal models (5–8). The molecular mechanism for the ability of NSAIDs to inhibit tumor formation is not known. However, most NSAIDs inhibit both COX-1 and COX-2 (9). The COX-1 enzyme, which is constitutively expressed in nearly all tissues in the body, predominantly plays a housekeeping role by mediating normal physiologic processes such as protection of the stomach and platelet aggregation (10). The other isoform, COX-2, is inducible by various mitogens, growth factors, and mediators of inflammation in certain cells and tissues (11).

Although COX-1 is constitutively present in normal colon tissue, COX-2 levels are low to undetectable. In contrast, COX-2 mRNA is up-regulated in tumor tissue, and COX-2 protein is expressed in high amounts (12). In rats that have been subjected to carcinogens, COX-2 mRNA and protein levels were increased in the tumors (13). Furthermore, cells overexpressing COX-2 have an altered cellular adhesion pathway, fail to undergo apoptosis, have increased invasiveness, and cell cycle prolongation (14–16). All of these changes were reversed by the addition of an NSAID, suggesting that COX-2-derived metabolites play an important role in tumorigenesis (14–16). In animal models, the absence of COX-2 expression inhibited polyp formation to 84% of control (17), and overexpression of the COX-2 gene in transgenic mice was sufficient to induce polyp formation in >85% of the mice (18).

Arachidonic acid is the preferred substrate for COX-2. It catalyzes the bis-dioxygenation of arachidonic acid to form PG\textsubscript{2}\textalpha, a cyclic endoperoxide with a hydroperoxide at C-15. This is followed by reduction of the C\textsubscript{15}-hydroperoxide to give PG\textsubscript{2}\textalpha (the peroxidase reaction). PG\textsubscript{2}\textalpha is the precursor to the formation of thromboxane A\textsubscript{2}, prostaclin, and other PGs (9, 19). When mice were fed eicosapentaenoic acid, an ω-3 fatty acid, this resulted in a significant reduction in intestinal arachidonic acid content and PGE\textsubscript{2} levels as well as a 64% reduction in polyp load (20). In addition, when cytosolic phospholipase 2, a phospholipase that has been well characterized as a major arachidonic acid releasing enzyme, was deleted in Min mice, there was an 83% reduction in polyp number (21).

The ability to inhibit polyp formation by NSAIDs and the reduction of arachidonic acid availability suggested that eicosanoid production may be very important in promoting carcinogenesis. In addition, eicosanoids have the ability to modulate various steps involved in carcinogenesis such as apoptosis and angiogenesis. However, eicosanoids are also produced by COX-1, which is present in both affected and unaffected tissue. This suggests that there may be a link between polyp formation and COX-2 up-regulation through a pathway that does not involve the biosynthesis of eicosanoids.

COX-2 can convert arachidonic acid into 15-hydroxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid (15-HETE) (22), which is derived from 15-HPTE, a prototypic ω-6 polyunsaturated fatty acid-derived lipid hydroperoxide. In settings of oxidative stress, where reducing pathways were compromised, 15-HPTE may survive long enough to induce DNA damage. Recently, it was demonstrated that 15-HPTE under-
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SCHEM E 1. Vitamin C-mediated decomposition of linoleic acid- and arachidonic acid-derived lipid hydroperoxides to bifunctional electrophiles and their corresponding substituted and unsubstituted etheno adducts. LOX, lipoxygenase; HPODE, hydroperoxyoctadecadienoic acid; DODE, 9,12-dioxo-10(E)-dodecenoic acid.

went homolytic decomposition to form DNA-reactive bifunctional electrophiles, HPNE, HNE, ONE, and EDE (23). Two distinct pathways of decomposition were identified. The first involved the intermediate formation of HPNE, and the second involved a series of complex rearrangements to EDE (24, 25). HPNE was shown to be the immediate precursor of ONE and HNE (22, 23, 26–28). It was recently demonstrated that HPNE also formed unsubstituted etheno adducts with double-stranded DNA (29). EDE also formed unsubstituted etheno adducts (25), and ONE formed heptanone-etheno adducts (30–32) (Scheme 1). However, HPNE was 10 times more efficient in the formation of unsubstituted etheno adducts than EDE, suggesting that it is the primary source for the formation of these adducts (29).

Unsubstituted etheno adducts are highly mutagenic in mammalian cells and have been detected in human DNA (33, 34). Recently, we showed that the ONE-derived heptanone-etheno adduct, HNE, was highly mutagenic in both bacteria and human cells.3 Interestingly, there were substantial differences in mutational frequency and base substitution between bacteria and human cells.3 In earlier studies we showed that vitamin C induces the decomposition of lipid hydroperoxides to DNA-reactive bifunctional electrophiles. This made it possible to develop an in vitro system to show that DNA damage occurred in rat intestinal epithelial cells stably expressing COX-2. There was a dose-dependent increase in HedGuo adduct formation in the presence of vitamin C in these cells (35). HedGuo adduct formation together with 15(S)-HPETE biosynthesis was inhibited by a specific COX-2 inhibitor (35). Therefore, there is now substantial evidence that COX-2-mediated DNA damage may play a role in tumorigenesis.

Min mice are one of the most commonly used mouse models for colorectal cancer. These mice spontaneously develop large numbers of polyps. In addition, up-regulation of COX-2 in the small intestine in these mice has been well characterized (36). Therefore, this mouse model and its wild type, non-COX-2 expressing counterpart were used to examine whether heptanone-etheno adducts were present in the DNA. A quantitative stable isotope dilution LC/ESI/MS/MS assay was used to quantify heptanone-etheno adducts in the small intestinal tissue.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Ammonium acetate, activated calf thymus, vitamin C, DNase I, and zinc chloride were purchased from Sigma-Aldrich. 15(S)-HPETE was obtained from Cayman Chemical (Ann Arbor, MI). High performance liquid chromatography grade water and acetonitrile were obtained from Fisher. Nuclease P1 and shrimp alkaline phosphatase (SAP) were obtained from Roche Diagnostics.13C15N-La
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Animals—C57BL/6 and C57BL/6 APCmin mice were obtained as gifts from Dr. Colin Funk of Queen’s University, Kingston, ON, Canada. Mice were fed ad libitum and sacrificed by CO2 asphyxiation. The entire small intestine was removed and immediately frozen at −80 °C until required for DNA extraction.

Mass Spectrometry—Mass spectrometry for the quantitative analysis of five DNA adducts was conducted with a Finnigan TSQ Quantum Ultra AM spectrometer (Thermo Electron Corp., San Jose, CA) equipped with an ESI source in the positive ion mode. Operating conditions were: spray voltage, 4.5 kV; heated capillary temperature, 350 °C. Nitrogen was used for the sheath gas and auxiliary gas set at 60 and 10 (in arbitrary units), respectively. Collision-induced dissociation was performed using argon as the collision gas at 1.5 milliTorr in the second (radio frequency-only) quadrupole. An additional direct current offset voltage was applied to the region of the mass analyser to aid in suppression of interference from solvent adduct ions. The spectrometer was operated in the multiple reaction monitoring (MRM) mode using a range of transitions monitored.

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**Liquid Chromatography—LC/UV chromatography** for quantitative DNA base analysis was conducted using gradient system 1 on a Hitachi L-2130 Intelligent Pump equipped with a Hitachi L2450 diode array detector (Hitachi, San Jose, CA). The separation employed a Phenomenex Jupiter 5-μm C18 column (250 × 4.6 mm inner diameter, 5 μm; Phenomenex, Torrance, CA). Solvent A was water, and solvent B was acetonitrile. The gradient was as follows: 0% B at 0 min, 0% B at 5 min, 8% B at 25 min, 100% B at 36 min, 0% B at 37 min, and 0% B at 52 min. The flow rate was 1 ml/min. The separation was performed at ambient temperature.

**LC/ESI/MS analysis** for DNA adducts on gradient system 2 was performed using a Hitachi L-2130 Intelligent Pump. The separation employed a Phenomenex Luna 3-μm C8 column (150 × 4.6-mm inner diameter, 3 μm). Solvent A was 5 mM ammonium acetate in water, and solvent B was 5 mM ammonium acetate in acetonitrile. The gradient was as follows: 6% B at 0 min, 6% B at 2 min, 9% B at 12 min, 55% B at 22 min, 80% B at 30 min, 80% B at 33 min, 6% B at 35 min, and 6% B at 45 min.

**TABLE 1**

|                  | HedCyd | HedGuo | HedAdo | HedCyd | HedGuo | HedAdo |
|------------------|--------|--------|--------|--------|--------|--------|
| C57BL/6J control mice | 0.10 ± 0.17 | 0.58 ± 0.40 | BDL | BDL | BDL | BDL |
| C57BL/6JAPC^min mouse | 1.07 ± 1.41 | 1.82 ± 1.25 | BDL | BDL | BDL | BDL |
| p value           | 0.04    | 0.01   |        |        |        |        |

The flow rate was 0.3 ml/min. The separation was performed at ambient temperature.

**Extraction of DNA from Mouse Tissue**—DNA isolation from the small intestine of mice was performed using a Wako DNA extraction WB kit (Wako Chemicals, Richmond, VA). Briefly, tissue was homogenized in lysis...
solution and then treated with an enzyme reaction solution, RNase, and protease to liberate the DNA from the nucleus. This was followed by NaI extraction and several wash steps to purify the DNA. Typically several mg of DNA were obtained. It was hydrolyzed as described below, and normal bases were quantified by LC/UV using system 1.

Hydrolysis of DNA and Isolation of DNA Adducts—The extracted DNA was combined and dissolved in Chelex-treated 10 mM Tris buffer (22) containing 100 mM MgCl₂ (pH 7.4). DNase I (556 units) was then added followed by alkaline phosphatase (30 units) in SAP 10 min at 37 °C. The samples were filtered through a 0.2-μm Costar cartridge. At this time an aliquot was removed for LC/UV analysis, parent ions were monitored for the protonated molecular ion and for its 15N5 or 15N3 internal standard. Adduct levels were normalized to the amount of DNA as detected by base analysis mentioned above.

Artifact Formation during Isolation and Analysis—Duplicate small intestine samples from Min mice were spiked with [13C15N]-labeled DNA (520 μg) before defrosting. The amount of unlabeled DNA in each sample was estimated as 650 μg. Labeled and unlabeled DNA were extracted from the tissues, hydrolyzed in the presence of 15N internal standards using DNase, nuclease P1, and alkaline phosphatase. DNA adducts were isolated by solid phase extraction as described above. The samples were then analyzed by LC/MS using gradient system 2. The 15N5- or 15N3-labeled internal standards were added to the samples before hydrolysis. Quantitation was performed from standard curves constructed by the ratio of known amounts of authentic standards and internal standards. For the MRM analysis, parent ions were monitored for the protonated molecular ion of the endogenous adduct and for its 15N5 or 15N3 internal standard. Product ions that were monitored corresponded to the loss of the 2'-deoxyribose moiety (BH₂⁺) from the endogenous adduct and its 15N5 or 15N3 internal standard. Adduct levels were normalized to the amount of DNA as detected by base analysis mentioned above.
RESULTS

**MS Analysis of DNA Adducts**—Chromatograms for and CdgGuo, CdgCyd, HedoAdo, HedoCyd, and HedoGuo derived from LC/ESI/ MRM/MS analysis of a five-DNA adduct standard mixture, and their corresponding heavy isotope internal standards are shown in Figs. 1 and 2. Standard curves were constructed for each adduct in the range of 0.01 to 2 pg/ml, and control samples were analyzed. A typical regression line for CdgCyd, HedoCyd, HedoGuo, and CdgGuo was y = 0.005x − 0.0138 (r² = 0.9958), y = 0.0051x + 0.0019 (r² = 0.9997), y = 0.0042x + 0.0026 (r² = 0.9989), y = 0.001x + 0.0018 (r² = 0.998), and y = 0.0052x − 0.0015 (r² = 0.995), respectively.

**MS Analysis of C57BL/6Mice**—DNA was isolated from the entire small intestine of C57BL/6 mice. The DNA was subjected to enzyme hydrolysis in the presence of 15N5-labeled HedoGuo (m/z 640 → m/z 288), 15N3-labeled HedoGuo (m/z 419 → m/z 298; from labeled DNA), 15N3-labeled HedoAdo (m/z 409 → m/z 293; internal standard). B, ion chromatograms for endogenous CdgCyd (m/z 368 → m/z 272), 15N3-labeled HedoAdo (m/z 403 → m/z 282; from labeled DNA), 15N3-labeled HedoAdo (m/z 393 → m/z 277; internal standard). C, ion chromatograms for endogenous CdgCyd (m/z 364 → m/z 248), 15N3-labeled HedoCyd (m/z 376 → m/z 255; from labeled DNA), 15N3-labeled HedoCyd (m/z 367 → m/z 251; internal standard).

**DISCUSSION**

Over the last two decades there has been a substantial effort to identify lipid hydroperoxide-derived endogenous DNA adducts as potential biomarkers for cancer. Using highly specific and sensitive stable isotope dilution methodology in combination with analysis by gas chromatography/MS or LC/MS, it has been possible to quantify eGua, edGua, and HedoCyd in the presence of calf thymus DNA (5 mM) for 24 h at 37 °C. The reaction was placed on ice for 15 min. DNA was precipitated by cold ethanol and 2.9 mM sodium acetate. It was hydrolyzed as described above, and normal bases were quantified by LC/UV using system 1.

**FIGURE 8.** LC/ESI/MMR/MS analysis of DNA adducts from the small intestine of a Min mouse spiked with 13C15N-labeled DNA (520 µg). The amount of unlabeled DNA was estimated as 650 µg based on the amount of tissue used. A, ion chromatograms for endogenous HedGuo (m/z 640 → m/z 288), 15N5-labeled HedGuo (m/z 591 → m/z 288; from labeled DNA), 15N3-labeled HedGuo (m/z 409 → m/z 293; internal standard). B, ion chromatograms for endogenous HedAdo (m/z 368 → m/z 272), 15N3-labeled HedAdo (m/z 403 → m/z 282; from labeled DNA), 15N3-labeled HedAdo (m/z 393 → m/z 277; internal standard). C, ion chromatograms for endogenous HedCyd (m/z 364 → m/z 248), 15N3-labeled HedCyd (m/z 376 → m/z 255; from labeled DNA), 15N3-labeled HedCyd (m/z 367 → m/z 251; internal standard).

**FIGURE 9.** Amount of DNA adducts (adducts/10^6 normal bases) formed in the calf thymus DNA treated with 15-HPETE and vitamin C. Determinations were conducted with an n = 3 (means ± S.E.). White bars, 15-HPETE-treated calf thymus DNA; black bars (not visible), untreated calf thymus DNA.
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A schematic representation of the biological processes involved in the formation and detection of endogenous DNA adducts in Min mice is presented. The text explains the methods used for detection, including the use of mass spectrometry and immunofluorescence techniques. The results indicate that certain adducts, such as M1G, are more abundant in the Min mice compared to the wild type mice, suggesting a correlation with oxidative stress and DNA damage.

**Table 2: Endogenous DNA adducts quantified in mammalian tissues using stable isotope dilution MS**

| Adduct | Method | Species | Source | Ref. |
|--------|--------|---------|--------|------|
| M1G    | Immuno-extraction LC/APCI/MS | Human | Urine | 47 and 48 |
|        | GC/ECNCI/MS | Human, Rat | Liver, leukocytes | 49–51 |
|        | LC/ESI/MS | Human | Leukocytes | 51 |
| dAdo   | Immuno-extraction LC/ESI/MS | Rat | Urine, lung, liver | 53 and 54 |
|        | GC/ECNCI/MS and LC/ESI/MS | Human | Placenta, urine | 55–58 |
|        | LC/APCI/MS | Human | Urine | 59 |
| dCyd   | Immunoaffinity-LC/ESI/MS | Human | Urine | 60 |
|        | GC/ECNCI/MS | Rat | Liver | 61 and 62 |
|        | LC/ESI/MS | Human | Urine | 63 |
|        | LC/ESI/MS | Human | Urine | 64 |
|        | LC/ESI/MS | Rat | Liver | 65 |

In summary, we have detected the endogenous formation of heptanone-etheno DNA adducts in Min mice DNA. There were statistically significant increased levels of the adducts that we were able to quantitate in the C57BL/6JAPC<sup>min</sup> mice versus the C57BL/6J mice (Table 2 and Fig. 5). These data correlate with our cell culture experiments (35), showing that increased levels of COX-2, as is the case in the small intestine of the C57BL/6JAPC<sup>min</sup> mice (36), result in increased levels of the adducts. H<sub>E</sub>dGuo was increased from 0.6 adducts/10<sup>7</sup> normal bases to 1.8 adducts/10<sup>7</sup> bases (Table 1). H<sub>E</sub>dCyd was also increased from 0.1 adducts/10<sup>7</sup> normal bases to 1.1 adducts/10<sup>7</sup> normal bases (Table 1).

The peaks for CedGuo and CedCyd were in the range of noise and close to the zero point of the assay, making it impossible to accurately quantify any adducts that may have been present (Figs. 3 and 6). H<sub>E</sub>dAdo levels were close to the lower limit of detection, making the analysis difficult (Figs. 4 and 7). However, there appeared to be a trend toward an increase in the Min mice.

These data suggest that the H<sub>E</sub>dGuo and H<sub>E</sub>dCyd adducts may be good markers for the detection of colorectal cancer. The major adducts identified in calf thymus DNA after treatment with 15(S)-HPETE were the heptanone-etheno adducts. H<sub>E</sub>dGuo was increased from 0.6 adducts/10<sup>7</sup> normal bases to 1.8 adducts/10<sup>7</sup> bases (Table 1). H<sub>E</sub>dCyd was also increased from 0.1 adducts/10<sup>7</sup> normal bases to 1.1 adducts/10<sup>7</sup> normal bases (Table 1).

In summary, we have detected the endogenous formation of heptanone-etheno DNA adducts for the first time in mammalian tissue. Using highly specific and sensitive LC/ESI/MRM/MS methodology, it was possible to quantify the amount of adducts present in the colon tissue of normal C57BL/6J mice and C57BL/6JAPC<sup>min</sup> mice, a colorectal cancer model. There were statistically significant increased levels of adducts in the Min mice when compared with the wild type mice. This suggests that heptanone-etheno adducts in colon tissue DNA may serve as a biomarker of increased risk for colorectal cancer. Furthermore, there was a difference in the predominant adducts formed between an in vitro system and the in vivo mouse model, which suggests that DNA adducts are formed in vivo.
repair enzymes may favor certain DNA adducts. The adducts that are efficiently repaired may ultimately be excreted in the urine. Analysis of these adducts may provide biomarkers of endogenous lipid hydroperoxide-mediated DNA damage in a similar manner to the well characterized urinary DNA adducts of aflatoxin that have served so well as biomarkers of aflatoxin-mediated DNA damage (52).

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