New Plausible Mechanism for Gastric and Colorectal Carcinogenesis: Free Radical-Mediated Acetaldehyde Generation in a Heme/Myoglobin–Linoleate–Ethanol Mixture

Hiroshi Kasai* and Kazuaki Kawai

ABSTRACT: Epidemiological studies have revealed that alcohol, red meat, and cooking oil (or linoleate) are risk factors for both gastric and colon cancers. A survey of the mutation spectra of the p53 tumor suppressor gene in these cancers suggested that the types of mutations and the hot spots are similar to those induced by acetaldehyde (AcAld) in an in vitro p53 mutation analysis system. Accordingly, various combinations of possible factors, components, or model compounds were reacted in an emulsion and tested for the generation of AcAld. Efficient AcAld formation was only observed with combinations of three factors, red meat homogenate (or heme/myoglobin), methyl linoleate, and ethanol, but not by any combination of the two. The generated AcAld levels (ca. 500 μM) far exceeded the minimum mutagenic concentration (40–100 μM) obtained using concentrations of meat homogenate (or heme/Mb), linoleate, and ethanol comparable to those in the stomach after an ordinary meal. A mutagenic level of AcAld (75 μM) was also generated with a physiological concentration of ethanol, heme, and linoleate in the colon. As a mechanism, linoleate hydroperoxide formation and its decomposition in the presence of myoglobin (or heme) to generate the OH radical seem to be involved in the ethanol-to-AcAld conversion.

INTRODUCTION

Gastric and colorectal cancers are the second and third most common causes of cancer deaths, respectively. Epidemiological studies have shown that the consumption of alcohol and red meat, especially processed meat, are risk factors for both gastric and colon cancers. Alcohol consumption induces gastrointestinal tract (GI tract) cancers, including oral, esophageal, gastric, and colorectal cancers, by alcohol dehydrogenase (ADH)-mediated acetaldehyde (AcAld) generation in these tissues or bacterial flora. On the other hand, in relation to red meat-induced colorectal carcinogenesis, the formation of N-nitroso compounds or lipid peroxidation products, such as malondialdehyde and 4-hydroxynonenal, triggered by the heme (Fe) in red meat has been suggested as possible mechanisms. Fecal metabolomic analyses have revealed the strong association between fecal heme-related molecules and colorectal cancer. In addition, the frequent use of cooking oil, especially its reuse for frying, was significantly associated with gastric cancer risk. An increased colorectal cancer risk is also correlated with the intake of fried foods, such as French fries, fried chicken, and fried fish, from fast-food restaurants. Linoleate intake is reportedly correlated with K-ras mutated colon cancer, and ethyl linoleate stimulated dimethylhydrazine-induced colon carcinogenesis in rats.

The most frequent mutations of the p53 tumor-suppressor gene found in gastric and colon cancer are GC → AT at CpG sites, which are frequently methylated. The mutation spectra and the hot spots in the p53 gene are similar to those induced by AcAld in an in vitro assay system using a plasmid containing the p53 gene. In fact, AcAld induces interstrand GG crosslinks specifically at CpG sites, leading to G → A transitions. These results strongly suggested that AcAld is a plausible cause of these cancers.

AcAld was implicated as a carcinogenic ethanol metabolite involved in GI tract carcinogenesis. Various alcoholic beverages also contain mutagenic levels of AcAld. The International Agency for Research on Cancer classified AcAld as "possible carcinogenic to humans (group 2B)" in 1999. Subsequently, AcAld in association with alcohol consumption was upgraded to group 1 (the highest level of evidence) as "carcinogenic to humans."

Based on this information, we examined the formation of AcAld by chemical mechanisms from the components of these risk factors: alcohol, red meat, and cooking oil. This study focused on AcAld generation by the reaction of these three
factors in an emulsion because their consumption may often co-occur in daily dietary habits.

■ RESULTS

Analysis of AcAld. Based on the previous reports, we initiated preliminary experiments to search for the optimal conditions of AcAld formation within a short reaction time. The pH 4.5 condition was selected from ref 21. The solution of DNPH in acetonitrile was used for the derivatization, according to the method described in ref 22. We found that the reaction conditions [0.2 M acetate buffer (pH 4.5): acetonitrile (1:1)] with excess DNPH (3 mM) were suitable for our purpose, in that the reaction is completed within 3 min (Figure S2, Supporting Information). The short reaction time is important to avoid the artifactual formation of AcAld during derivatization. Our results were similar to those reported by Kozutsumi et al. in which the reaction conditions including acetate buffer (0.1 M, pH 4), dimethyl sulfoxide (36%), and DNPH (2 mM) were used, and the reaction was completed within 2 min at room temperature.

The chromatograms of the analysis of AcAld formed in a hemin−methyl linoleate (MLA)−ethanol mixture are shown in Figure 1. The hydrazone products produced by the reaction of AcAld with DNPH were eluted as a mixture of E- and Z-stereoisomers, as detected by the UV absorbance at 360 nm, and quantified against the standard. The retention time and UV spectrum of the product in high-performance liquid chromatography (HPLC) were identical to those of the standard AcAld treated with DNPH. The identity of the AcAld−DNPH derivative was further confirmed by liquid chromatography/mass spectrometry (LC/MS) (Figure 2).

Formation of AcAld in the Model Reactions. Various model reactions were examined at pH 4.5 and pH 7.4 as representative pH values of the gastric juices of normal and high-risk groups, respectively (further details in the Discussion). The experiments at pH 7.4 also correspond to the pH of the colonic contents of colon cancer high-risk groups. As an initial condition of digestion in the stomach, the meat reaction was also examined with pepsin at pH 3.0. A significant increase in the AcAld concentration (210−470 μM) was observed in the hemin + MLA + EtOH reactions at pH 4.5 and pH 7.4 (Figure 3). The yield of AcAld was higher at pH 4.5.

Figure 1. HPLC analysis of AcAld derivatized with DNPH. (a) Standard, (b) hemin−MLA−ethanol reaction mixture, and (c) blank. Arrows indicate the AcAld−DNPH peak. Insets are UV spectra of the arrow peaks.
than that at pH 7.4. The increase was less than 70 μM in the combinations of two components, such as hemin + MLA, MLA + EtOH, or hemin + EtOH, irrespective of the pH. The reaction mixtures (hemin + MLA, pH 4.5, pH 7.4) generate AcAld to some extent without ethanol. Small carbonyl compounds including AcAld may be formed by lipid peroxidation via sequential changes, L → LOOH → alkoxyl radical → β-scission → aldehydes. In fact, urinary AcAld is measured as a biomarker of lipid peroxidation in rats. When meat homogenate or myoglobin (Mb) was used instead of hemin, the reactions of raw meat, salami, and Mb generated high levels of AcAld (260−470 μM) (Figures 4, 5). In the meat and Mb experiments, AcAld formation was higher at pH 7.4 than at pH 4.5 (Figure 4). In the reaction with meat, salami, and Mb, some of the generated AcAld may react with proteins to form mainly unstable, reversible adducts and small amounts of stable adducts. The tendency of the gradual
Organic hydroperoxide and \( \text{H}_2\text{O}_2 \) are reportedly oxygen formation was observed after 23 h incubation (Figure 6). The human colon after alcohol and meat intake, 75 experiments are plotted. Bars represent the two experimental values.

EtOH, pH 4.5; (● ●) hemin + MLA + EtOH, pH 7.4. Mean values of duplicate experiments are plotted. Bars represent the two experimental values.

Comparison of AcAld formation between raw and baked meat, salami, and heme. (X—X) Raw meat + MLA + EtOH, pH 7.4; (■ ■) salami + MLA + EtOH, pH 7.4; (● ●) hemin + MLA + EtOH, pH 4.5; (○—○) baked meat + MLA + EtOH, pH 7.4; (◆ ◆) hemin + MLA + EtOH, pH 7.4. Mean values of duplicate experiments are plotted. Bars represent the two experimental values.

Figure 5. Comparison of AcAld formation between raw and baked meat, salami, and heme. (X—X) Raw meat + MLA + EtOH, pH 7.4; (■ ■) salami + MLA + EtOH, pH 7.4; (● ●) hemin + MLA + EtOH, pH 4.5; (○—○) baked meat + MLA + EtOH, pH 7.4; (◆ ◆) hemin + MLA + EtOH, pH 7.4. Mean values of duplicate experiments are plotted. Bars represent the two experimental values.

Organic Hydroperoxide Stimulates AcAld Formation.

Organic hydroperoxide and \( \text{H}_2\text{O}_2 \) are reportedly oxygen donors in a P450-mimic-mutagen activation system.\(^{29}\) To clarify the mechanism of AcAld formation in the above-mentioned model reactions, \( t\text{BuOOH} \) was added to the Mb + EtOH mixture at pH 7.4. Time (0–20 min) - and \( t\text{BuOOH} \) dose (250 \( \mu \text{M} \) to 4 \( \mu \text{M} \))-dependent AcAld formations were observed, as shown in Figure 7a, respectively. When a high concentration (45 \( \mu \text{M} \)) of \( \text{H}_2\text{O}_2 \) or \( t\text{BuOOH} \) was used in the reaction, 40-fold lower AcAld formation was observed with \( \text{H}_2\text{O}_2 \) as compared to that detected with \( t\text{BuOOH} \) (data not shown).

### DISCUSSION

Chemistry-based studies are important to clarify the molecular mechanisms of carcinogenesis deduced from epidemiological data.\(^{30}\) This is the first report of AcAld formation by chemical reactions from three dietary components, meat/Mb/heme, linoleate, and ethanol, which are known risk factors for gastric and colon cancers. AcAld has been frequently discussed as a cause of alcohol-induced cancers. However, its role in red meat-induced colorectal cancers has not been reported so far. In the present study, high concentrations of AcAld up to 500 \( \mu \text{M} \) were produced in the model reactions. This level of AcAld markedly exceeds the minimum mutagenic concentration (MMC, 40–100 \( \mu \text{M} \)). The AcAld level was much higher than that formed by bacteria in the stomach (55.4 \( \mu \text{M} \)) under the conditions of hypochlorhydria after alcohol ingestion (0.6 g/kg).\(^{31}\) The level of AcAld (75 \( \mu \text{M} \)) produced in the model reaction, with the physiological concentrations of heme (160 \( \mu \text{M} \)) and ethanol (22 \( \mu \text{M} \)) in the colon, was comparable to that generated by human colonic bacteria (ca. 110 \( \mu \text{M} \)) by incubation with 22 \( \mu \text{M} \) ethanol.\(^{27}\) Rose et al.\(^{28}\) reported that the fecal heme concentration exceeds 3 \( \text{mg/g} \) (200 \( \mu \text{M} \), calculated as fecal density 1.075)\(^{32}\) in 49% of the human subjects after a meat challenge diet (250 \( \text{g/day} \)). Therefore, the heme reaction (160 \( \mu \text{M} \)) data in the present study may underestimate the AcAld generation in the colon.

The concentrations of hemin, meat (calculated as heme), and alcohol used in these reactions are similar to the ranges in ordinary meals. For example, in the heme experiments, 60 or 160 \( \mu \text{M} \) heme corresponds to the consumption of 60 or 170 g meat, respectively, if the Mb content in meat is 1% and the digestion volume in the stomach is tentatively 500 mL. In the meat experiments, 60 \( \mu \text{M} \) heme equivalent corresponds to the consumption of 60 g of meat. The amount of ethanol (1.4 M) in the model reactions corresponds, for example, to 350 mL of wine per 500 mL of digestion volume in the stomach. The concentration of MLA (ca. 3%) in the reaction mixtures is similar to the range that stimulated colon carcinogenesis in rat experiments (ca. 5%).\(^{12}\)

In the present study, higher levels of AcAld were formed by raw meat and salami, which was made by a low-temperature fermentation procedure as compared to the baked meat. The former two probably contain higher levels of intact Mb than baked meat. It is worth mentioning that not only raw meat and salami but also many meat preparations, such as roast beef and rare-medium steak, may contain intact Mb if the internal meat temperature is around 60 °C during cooking.

Various alcoholic beverages contain a wide range of AcAld concentrations. For example, beer and wine contain 210 and 474 \( \mu \text{M} \) AcAld on average, respectively.\(^{19}\) Direct exposure to high AcAld due to the consumption of these beverages may increase oral and esophageal cancer risks, but it will be diluted in the stomach. However, the AcAld generated in the stomach saturation of AcAld formation in the meat, salami and Mb reactions (Figures 4, 5) in the latter period may be partly due to AcAld–protein adduct formation. Under the pH 3.0 conditions with pepsin, the AcAld formation was low (Figure 4). Baked meat had lower activity than raw meat (Figure 5). Free hemin showed 3–5-fold lower activity than raw meat, salami, and Mb, when the molar concentration of heme was adjusted to be the same (60 \( \mu \text{M} \), Figure 5). With physiological concentrations of ethanol (22 \( \mu \text{M} \))\(^{27}\) and heme (160 \( \mu \text{M} \))\(^{28}\) in the human colon after alcohol and meat intake, 75 \( \mu \text{M} \) AcAld formation was observed after 23 h incubation (Figure 6).

![Graph](https://doi.org/10.1021/acsomega.1c00614)

**Figure 6.** AcAld formation by physiological concentrations of EtOH (22 \( \mu \text{M} \)), heme (160 \( \mu \text{M} \)), and MLA (3%). Mean values of duplicate experiments are plotted. Bars represent the two experimental values.
would be increased to 300–500 μM or more and maintained over several hours after the meal, if not decomposed by aldehyde dehydrogenase. This may be more dangerous than the intrinsic AcAld in the beverages.

In high-risk gastric cancer groups, such as achlorhydric patients, especially those with pernicious anemia, hypogammaglobulinaemia, or gastric ulcers associated with *Helicobacter pylori* infection, the pH values of the gastric juice were around neutral (pH 6.5–8.1), including periods of fasting and digestion, while those in healthy people were pH 4–5 during the digestion period after the meal. Meat digestion may be slower at a higher stomach pH because pepsin would be inactive. Especially, Mb has a rigid structure and is not efficiently digested. In mice, a portion of undigested Mb reportedly reaches the colon after meat intake. A high-risk colon cancer group reportedly had a fecal pH of 7.0–8.0. Therefore, the high AcAld formation in the meat and the Mb reactions at pH 7.4 may be related to both gastric and colorectal carcinogenesis.

The data in Figure 7 suggested that organic hydroperoxide is a key intermediate for AcAld formation. It may be produced during cooking or in the GI tract during digestion. Increases of lipid hydroperoxides in cooking oils during frying cycles, measured as peroxide values, have been reported. In a stomach model, linoleic acid hydroperoxide (LOOH) is reportedly generated in an emulsion reaction mixture (pH 3) containing meat/metMb/Fe³⁺ and linoleic acid in the presence of oxygen. In rat experiments, a diet with high Fe³⁺ plus 15% corn oil induced higher LOOH in feces. A 13-fold greater amount of OH radicals is reportedly generated in human feces after a high meat and fat diet as compared to that after low meat and fat diet. The OH radical generated by LOOH decomposition seems to be involved in the mechanism of ethanol-to-AcAld conversion. In support of this conclusion, Rota et al. reported that the reaction of a heme-protein cytochrome P450 with LOOH generates a OH radical based on electron spin resonance studies. The production of OH radicals was also observed by the reaction of LOOH with FeSO₄ (Fe²⁺) or FeCl₃ (Fe³⁺). Therefore, AcAld formation via a OH radical in the presence of ethanol can apparently occur in both the stomach and colon after a meal.

Although the ratios of free heme and intact Mb in the stomach and colon after meat consumption are not known, in many of the model reactions containing heme/Mb/meat, ethanol, and MLA, especially at pH 7.4, the AcAld concentration exceeded the MMC and seemed to be related to stomach and colorectal carcinogenesis. In support of this mechanism, epidemiological studies suggested that the co-consumption of alcohol and red meat synergistically increases the colon cancer risk. It is also worth mentioning that the gastric cancer risk is significantly enhanced by the interaction of *H. pylori* infection (high gastric pH) and red meat intake.

In conclusion, when considering sources of AcAld, we must be careful to assess its formation from dietary components in the stomach and colon by a free radical mechanism, in addition to alcohol metabolism by ADH and the intrinsic AcAld levels in alcoholic beverages. If the main cause of gastric and colorectal cancers is the AcAld generated by the mechanisms presented in this paper, then it is possible to provide strategies for the prevention of these cancers, for example, by avoiding the simultaneous consumption of red meat and alcohol or by eating vegetables and fruits that contain OH radical scavengers. In addition, the previously reported cysteine (Cys) tablet may also decrease the exposure to AcAld either by trapping OH radicals or by AcAld–Cys adduct formation. Further studies on the relationships between these dietary factors and AcAld–DNA adduct formation in GI tracts in animal experiments and their roles in carcinogenesis are required to prove this mechanism.

### EXPERIMENTAL SECTION

**Materials.** Hemin was purchased from Sigma-Aldrich Chemical Co., USA. Tween 20 was obtained from ICN Biochemicals Inc., USA. Horse Mb was procured from SERVA Electrophoresis GmbH, Germany. MLA was purchased from Tokyo Chemical Industry Co., Ltd. Japan. DNPH, ethanol (99.5%), *t*-butyl hydroperoxide (*t*BOOH) (70%), and hydrogen peroxide (30%) were obtained from Wako Pure Chemical Industries Ltd., Japan. Beef and salami were purchased in a grocery store.
Preparation of Meat Homogenates. Meat was baked in a pan without cooking oil until it was browned, as in the usual preparation. Portions (0.7 g) of raw and baked meat or salami were cut into small pieces and homogenized in water (5 mL) containing Tween 20 (10 μL, 0.2%) with a Polytron PT10-35 (Kinematica, Switzerland) homogenizer for 30 s at room temperature. The homogenates were divided into 500 μL aliquots in Eppendorf tubes (2 mL) and kept in a freezer at −20 °C until use.

Reaction of Hemin–MLA–EtOH (Method 1). Hemin was dissolved in 20 mM NaOH (2.17 mg/mL). The hemin solution (23 μL; final concentration, 160 μM), ethyl acetate (50 μL), MLA (20 μL), ethanol (50 μL), and 0.2 M sodium acetate buffer (400 μL, pH 4.5) were mixed in an Eppendorf tube (2 mL). The tube was capped and vigorously shaken to produce a homogeneous emulsion at 37 °C. The reaction was continued for 4 h. For the reaction at pH 7.4, 0.2 M phosphate buffer (pH 7.4) was used instead of the acetate buffer. These conditions were also used for the reactions of two components. In method 1, the addition of ethyl acetate was necessary to make a homogeneous emulsion because hemin is practically insoluble in the buffer. For comparisons between meat-, salami-, Mb-, and hemin-reactions, Tween 20 detergent was added to facilitate emulsion formation (method 2).

Reaction of Hemin–MLA–EtOH (Method 2, for Comparison with Meat Reactions). The hemin solution (11 μL, 60 μM), MLA (20 μL), ethanol (50 μL), and 0.2 M sodium acetate (pH 4.5) or phosphate (pH 7.4) buffer (546 μL) containing 0.2% Tween 20, were mixed in an Eppendorf tube (2 mL). The tube was capped and vigorously shaken to produce a homogeneous emulsion at 37 °C.

Reaction of Meat–MLA–EtOH. MLA (20 μL, 2 M phosphate buffer (pH 7.4) or acetate buffer (pH 4.5) (55 μL), and ethanol (50 μL) were added to the meat homogenate aliquots (500 μL) in an Eppendorf tube (2 mL). One of the raw meat homogenate aliquots (500 μL) was mixed with MLA (20 μL), ethanol (50 μL), 1 M NaH2PO4 (55 μL), and pepsin (2.0 mg), and the final pH was adjusted to 3.0 by adding 2 N HCl (ca. 7.5 μL). The tube was capped and vigorously shaken to produce a homogeneous emulsion at 37 °C. In these reactions, the approximate heme concentration was 60 μM based on the assumption that meats contain 1% Mb.

Reaction of Mb–MLA–EtOH. A 500 μL aliquot of the Mb solution (1.3 mg/mL water plus 2 μL Tween 20) (final concentration, 60 μM) was mixed with ethanol (50 μL), MLA (20 μL), and 2 M acetate (pH 4.5) or phosphate (pH 7.4) buffer (55 μL) in an Eppendorf tube (2 mL). The tube was capped and vigorously shaken to generate a homogeneous emulsion at 37 °C.

Reaction of Mb–BuOOH–EtOH. For the dose-dependent AcAld formation experiments, the solutions of Mb (100 μL), ethanol (10 μL), 2 M phosphate buffer (pH 7.4) (11 μL), and various concentrations of BuOOH (7.2 μL) were mixed in Eppendorf tubes and incubated at 37 °C for 20 min. For the time course experiment, the same conditions as above were used, except that a lower amount of BuOOH (final concentration, 260 μM) was added to the mixture.

Analysis of AcAld in the Reaction Mixture. AcAld was analyzed by a modified method based on the procedures by Guan et al.21 Madden et al.22 and Kozutsumi et al.23 Briefly, after centrifugation of the emulsion reaction mixture, 10 μL of the supernatant (or water for blank) was mixed with 100 μL of 0.2 M sodium acetate (pH 4.5) and 100 μL of DNPH solution in acetonitrile (1.25 mg/mL) and reacted for 3 min at room temperature (23 °C). After centrifugation, a 50 μL portion of the supernatant was immediately injected into an HPLC column (CAPCELL PAK C18 MG II, 3 μm, 4.6 × 150 mm, Shiseido Fine Chemicals, Japan) connected with a photodiode array UV detector (Hewlett-Packard 1100 HPLC detection system). The following linear gradient of acetonitrile concentration in 10 mM ammonium formate was used: 0–15 min, 50–100%; 15–20 min, 100%. The elution speed was 0.8 mL/min. The blank value was subtracted from each analysis value of the reaction mixture. The AcAld concentration was determined based on the calibration curve (Figure S1, Supporting Information).

LC/MS. AcAld–DNPH was identified by HPLC coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive Focus, Thermo Fisher Scientific, Waltham, MA) with negative-ion electrospray ionization-MS. The sample separation was achieved on an Acclaim 120 C18 column (2.1 mm × 50 mm, 3 μm, Thermo Fisher Scientific, Waltham, MA) with a flow rate of 0.3 mL/min and a column temperature of 30 °C. Mobile phase A was 10 mM ammonium formate, and mobile phase B was acetonitrile. The percentage of solvent B changed as follows: 0–2 min, 40%; 2–10 min, 40–90% (linear gradient). The injection volumes for the measurements were 5 μL. AcAld–DNPH (C8H8N4O4) was identified using the extracted ion chromatogram of m/z 223.04728 [M – H]−.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00614.

1. Calibration curve for the relation of peak area and AcAld concentration and time course of AcAld–DNPH formation (PDF)

AUTHOR INFORMATION
Corresponding Author
Hiroshi Kasai – Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu 8078555, Japan; orcid.org/0000-0003-0746-2585; Email: h-kasai@med.uoeh-u.ac.jp

Author
Kazuaki Kawai – Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu 8078555, Japan

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c00614

Notes
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