Chloroacetonitrile (CAN) Induces Glutathione Depletion and 8-Hydroxylation of Guanine Bases in Rat Gastric Mucosa

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ABSTRACT: Chloroacetonitrile (CAN) is detected in drinking-water supplies as a by-product of the chlorination process. Gastroesophageal tissues are potential target sites of acute and chronic toxicity by haloacetonitriles (HAN). To examine the mechanism of CAN toxicity, we studied its effect on glutathione (GSH) homeostasis and its impact on oxidative DNA damage in gastric mucosal cells of rats. Following a single oral dose (38 or 76 mg/Kg) of CAN, animals were sacrificed at various times (0–24 h), and mucosa from pyloric stomach were collected. The effects of CAN treatment on gastric GSH contents and the integrity of genomic gastric DNA were assessed. Oxidative damage to gastric DNA was evaluated by measuring the levels of 8-Hydroxydeoxyguanosine (8-OHdG) in hydrolyzed DNA by HPLC-EC. The results indicate that CAN induced a significant, dose- and time-dependent, decrease in GSH levels in pyloric stomach mucosa at 2 and 4 hours after treatment (56 and 39% of control, respectively). DNA damage was observed electrophoretically at 6 and 12 hours following CAN administration. CAN (38 mg/Kg) induced significant elevation in levels of 8-OHdG in gastric DNA. Maximum levels of 8-OHdG in gastric DNA were observed at 6 hours after CAN treatment [9.59 ± 0.60 (8-OHdG/10^5dG) 146% of control]. When a high dose of CAN (76 mg/Kg) was used, a peak level of 8-OHdG [11.59 ± 1.30 (8-OHdG/10^5dG) 177% of control] was observed at earlier times (2 h) following treatment. When CAN was incubated with gastric mucosal cells, a concentration-dependent cyanide liberation and significant decrease in cellular ATP levels were detected. These data indicate that a mechanism for CAN-induced toxicity may be partially mediated by depletion of glutathione, release of cyanide, interruption of the energy metabolism, and induction of oxidative stress that leads to oxidative damage to gastric DNA.

INTRODUCTION

Haloacetonitriles (HAN) are by-products of chlorination of drinking water (1,2). HAN were detected quantitatively in drinking-water samples (3). Previous reports have indicated that levels of HAN in drinking water should be lower than those currently proposed by WHO (4–6).

HAN induce adverse reproductive, mutagenic, and carcinogenic responses in animals (7–9). HAN induce sister chromatid exchange in Chinese hamster ovarian (CHO) cells and DNA strand breaks in cultured human lymphoblasts (10). Oral administration of HAN, such as chloroacetonitrile (CAN), bromoacetonitrile (BAN), and dibromoacetonitrile (DBAN), induces elevated levels of unscheduled DNA repair synthesis in gastric tissues (11).

The mechanism of CAN-induced genotoxicity was investigated (12,13). ^1^C-Chloroacetonitrile (^1^C-CAN) alkylates DNA in vivo, and the rate of DNA alkylation in maternal and fetal tissues of mice is GSH dependent (4). HAN are known to bind covalently to DNA in vitro to form N-7 cyanomethyl guanine adduct (14). CAN causes interruption of GSH homeostasis and inhibits glutathione transferase activities in liver and gastric tissues of rats (15).

GSH is important for the functions of gastroesophageal (GE) tissues (16), and depletion of GSH resulted in degeneration of the gastrointestinal epithelial cells. Preadministration of GSH or its precursors prevented such damages to GE tissues (16). Furthermore, the de-
pletion of GSH induces loss of mitochondrial functions and affects ATP production in the cells (17). Severe reduction in intracellular ATP causes loss of cellular homeostasis and viability (18,19). Previous reports indicates that mild ATP depletion results in chemical hypoxia, decrease in oxygen utilization, increase in oxygen tension, and formation of reactive oxygen species (ROS). Elevated levels of ROS induce oxidative damage to cellular constituents (20). It is imperative that, apart from its conventional role of detoxifying through conjugation of electrophilic species, GSH may play an additional role in protecting GI tissues against in situ generation of ROS and subsequent oxidative damage of macromolecules (21–23).

8-OHdG is used as a sensitive marker for oxidative DNA damage (24–29). This lesion is critical because unrepaired 8-OHdG becomes mutagenic during DNA replication (30–32). Furthermore, a direct correlation exists between 8-OHdG formation and carcinogenesis in vivo (33–36).

The objective of the current study is to investigate whether gastric mucosal tissues are capable of bioactivating CAN to reactive intermediates that deplete GSH and inhibit ATP production in the cells and also whether depletion of GSH and reduced levels of ATP lead to oxidative damage to gastric DNA.

**MATERIALS AND METHODS**

**Chemicals**

Chloroacetonitrile (CAN, purity >97%) was obtained from Aldrich (Milwaukee, WI). Standard 8-Hydroxy-2′-deoxyguanosine (8-OHdG) was purchased from Wako Bioproducts (Richmond, VA). 2′-Deoxyguanosine (dG), nuclease P1, and alkaline phosphatase were purchased from Sigma (St. Louis, MO). Proteinase-K, ribonuclease A, and deoxyribonuclease I were obtained from Worthington (Freehold, NJ). Buffers, HPLC solvents, and other chemicals used in this study were analytical grade or of highest purity commercially available.

**In Vitro Experiments**

**Gastric Mucosal Preparation and Incubation Mixtures**

Gastric mucosal suspensions were prepared for in vitro experiment by gentle scraping of rat pyloric mucosa with a sharp razor in ice cold phosphate buffer saline. Collagenase (1 mg/mL) was added, and the mucosal preparations were incubated for 30 minutes at 37°C in a shaking water bath. Unless otherwise indicated, each reaction mixture contained mucosal suspension, 10 μM Fe NH₃(SO₄)₂, and various concentrations of CAN (final volume, 3 mL). The reactions were started by the addition of CAN, and the incubations were carried out at 37°C for 1 hour. At the end of the incubation period, the reaction was terminated by freezing the mixtures at −30°C and maintained at this temperature until further analysis.

**Cyanide Determination**

Cyanide (CN⁻) concentrations in the incubation mixtures were determined electrochemically as described by Abreu and Ahmed (37). Briefly, 2 mL of each incubation mixture was added to 2 mL of 4 N H₂SO₄ in the outer chamber of a Conway microdiffusion cell, and 2 mL of 0.1 N NaOH was added to the inner chamber. The cells were sealed with a glass cover using silicone grease and rotated at 70 rpm for 2 hours using an orbital rotator. For electrochemical determination of cyanide ions, two electrodes connected to an Orion digital ion analyzer were placed in the center well of the Conway diffusion cell to which 0.05 mL of KAg(CN)₂ had been added as indicator. CN⁻ contents in the incubation mixtures were determined using KCN standard curve.

**ATP Determination**

ATP concentrations in the incubation mixtures were determined according to the method of Adams (38). The reaction of 3-phosphoglycerate and ATP catalyzed by phosphoglycerate kinase was coupled by the dephosphorylation reaction catalyzed by glyceraldehyde phosphate dehydrogenase that were involved in the oxidation of NADH. ATP levels were measured by assessing the oxidation of NADH to NAD.

**In Vivo Experiments**

**Animal Treatment**

Male Sprague–Dawley rats, 200–230 g (Charles River, Wilmington, MA), were acclimatized in our animal facility in 12 hours light/12 hours dark cycle for at least 3 days before the experiments. Animals were allowed food (Purina Lab Chow) and tap water ad libitum. Prior to treatment, animals were arranged in groups (4–5 rats/group) and given a limited amount of diet (4 g/rat) overnight to reduce their stomach contents, as complete starvation for more than 24 hours significantly depletes glutathione levels in gastrointestinal tissues (39).

For time-course studies, animals were administered a single oral dose of CAN (38 mg/kg) dissolved
in double-distilled water (0.2 mL) by gavage. The respective controls were given the vehicle (0.2 mL). Rats were decapitated at 2, 4, 6, 12, and 24 hours after treatment. For dose-response studies, a single dose of CAN (38 or 76 mg/kg) was orally administered. Rats were similarly sacrificed at 2 hours after treatment. Gastric mucosa was prepared by opening the stomach along its great curvature, washed in saline, and immediately frozen using dry ice. The mucosal preparations were stored at −80°C until further analysis.

Glutathione Determination

Frozen gastric mucosa (200 mg) from each rat were minced using a sharp razor blade and homogenized in sucrose (0.25 M). The homogenates were sonicated for 30 seconds in ice. GSH concentrations in homogenates were determined as described previously using Ellman’s reagent (4).

Isolation and Puriﬁcation of Gastric DNA

DNA was isolated from gastric mucosa by a modiﬁcation of previously described methods (40,41). Briefly, 100 mg of pyloric stomach mucosa was incubated with 1.2 mL digestion buffer (10 mM Tris HCl; 100 mM NaCl; 25 mM EDTA; 0.5% SDS, pH 8, containing 0.1 mg/mL proteinase K, and 10 µ/mL RNase) for 18 hours at 37°C. After digestion was complete, NaCl (5 M) was added to a ﬁnal concentration of 1 M followed by centrifugation at 4000 rpm for 15 minutes. The supernatant containing DNA was extracted twice with equal volumes of chloroform:isoamyl alcohol (24:1). DNA was precipitated from the upper aqueous phase by the addition of one-half volume of 7.5 M ammonium acetate and two volumes of ice cold ethanol. The mixture was gently shaken by inversion and kept at −20°C for 2 hours. Precipitated DNA was collected by centrifugation, washed with 70% ethanol, and resuspended in 10 mM Tris HCl (pH 7.4). The DNA thus obtained was found to be free from protein or RNA (purity 1.6–1.9) as quantiﬁed by standard spectrophotometric methods (42).

Gel Electrophoresis

DNA samples were resuspended in 10 mM Tris HCl containing 1 mM EDTA (pH 7.4) and mixed with agarose gel loading solution (0.05% bromophenol blue; 40% sucrose; 0.1 M EDTA pH 8; and 0.5% SDS) in a 5:1 ratio. Equal amounts (1 μg/20 μL) of DNA were loaded into a 1% agarose gel and electrophoresed, stained by 0.5 μg/mL ethidium bromide for 30 minutes, followed by destaining in water for 30 minutes. Photographs were taken using a still video system equipped with a strategene U/V transilluminator (La Jolla, CA).

DNA Digestion

Enzymatic hydrolysis of the oligonucleotides was carried out using previously described methods (40). Briefly, DNA samples were dissolved in 10 mM Tris-HCl (pH 7.4) and fragmented by vortex mixing and incubated with DNase I (40 units/100 µg DNA) in the presence of 10 mM Mg2+ at 37°C for 30 minutes. Sodium acetate (0.5 M) was added to optimize pH levels to pH 7.0 and Zn2+ (1 mM) and P1 nuclease (5 units/100 µg DNA) were added. The pH was readjusted to 7.5 with 0.4 M Tris-HCl (pH 7.5) buffer, and the mixture was incubated for 2 hours. Alkaline phosphatase (2.5 units/100 µg DNA) was added, and the mixture was incubated for an additional 30 minutes and cooled in ice. The enzymes were precipitated with 5 mL acetone (HPLC grade). Solvents were evaporated under reduced pressure at temperature not exceeding 30°C. The residues were redissolved in HPLC mobile phase and ﬁltered through a 0.2 µm membrane prior to HPLC analysis.

HPLC Analysis

The HPLC consisted of a model 580 dual piston pump and a model 5200A Coulochem II electrochemical detector (ESA, Bedford, MA). The potentials were set at +850 mV for the guard cell, +100 mV for the conditioning cell, and +500 and +800 mV for electrodes 1 and 2, respectively, for the analytical cell. 8-OHdG and dG were separated on a YMC B-02-3 C8 base-deactivated 3 µm, 4.6 mm × 15 cm column (YMC, Wilmington, NC) under isocratic conditions by a mobile phase containing 100 mM sodium acetate, and 5% methanol. The pH was adjusted to 5.2 with phosphoric acid (flow rate 1 mL/min). The chromatograms were collected and analyzed by chromjet dual-channel integrator (Spectra physics, Fremont, CA). Concentrations of 8-OHdG were expressed relative to the concentrations of dG.

Statistical Analysis

The GraphPAD (ISI Software, Philadelphia, PA) computer program was used to analyze and plot the data. Each experiment was conducted in triplicates, and results were expressed as mean ± S.E. of at least five determinations. The 0.05 level of probability was used as the criterion for signiﬁcance calculated using Student’s t-test.
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TABLE 1 Metabolism of CAN to CN\textsuperscript{-} by Gastric Mucosal Cell Suspension In Vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CAN (\mu M)</th>
<th>\text{CN}^{-} (\text{pmol/mg protein/min})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.8 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>160.3 ± 12.8</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>288.5 ± 9.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>333.3 ± 9.4</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>400.6 ± 6.3</td>
<td></td>
</tr>
</tbody>
</table>

Freshly prepared gastric cell suspension (0.6 mg protein/ml) was incubated with different concentration of CAN in presence of 10\mu M FeNH\textsubscript{4}(SO\textsubscript{4})\textsubscript{2}, at 37°C for 30 min. \text{CN}^{-} was determined as described in Materials and Methods. Data represents means ± SD of at least three determinations.

TABLE 2 Effect of Various Concentrations of CAN on ATP Levels in Gastric Mucosal Cell Suspensions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP (pmol/mg protein)</th>
<th>(% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.13 ± 0.98</td>
<td>100</td>
</tr>
<tr>
<td>CAN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM</td>
<td>8.57 ± 0.37\textsuperscript{a}</td>
<td>84.6</td>
</tr>
<tr>
<td>5 mM</td>
<td>6.75 ± 0.47\textsuperscript{a}</td>
<td>66.6</td>
</tr>
<tr>
<td>10 mM</td>
<td>4.68 ± 0.20\textsuperscript{a}</td>
<td>46.2</td>
</tr>
</tbody>
</table>

Freshly prepared gastric mucosal cell suspension (3.85 mg protein/ml) was incubated with different concentration of CAN at 37°C for 4 hours. ATP determinations were performed immediately as described in Materials and Methods. Data represents means ± SD of three determinations.

\textsuperscript{a}Significantly different from control.

RESULTS

Metabolism of CAN to CN\textsuperscript{-}

Incubation of CAN with gastric mucosal cell suspension resulted in the metabolism of CAN to CN\textsuperscript{-}. The data (Table 1) show that the 13–30-fold increase in the magnitude of CN\textsuperscript{-} release was dependent on CAN concentration. The data indicates that gastric mucosal cells are capable of CAN biotransformation.

Effect of CAN Exposure on ATP Levels

When gastric mucosal cell suspensions were exposed to various concentrations of CAN, significant (P < 0.05) decrease in the levels of gastric ATP was detected. The data in Table 2 indicate that CAN-induced ATP depletion was dependent on CAN concentration.

Effect of CAN on GSH Levels

To elucidate the effect of CAN on the gastric mucosal GSH contents in vivo, time-course and dose-response studies were conducted. In the time-course studies (Figure 1), the administration of a single oral dose of CAN (38 mg/kg) induced a significant decrease in GSH contents in rat gastric mucosa. The decrease in GSH contents was time dependent. At 2 hours after CAN administration, GSH levels decreased to 58% of control (1.79 ± 0.16 \text{\mu moles/g tissue}). The maximal decrease in GSH contents (1.42 ± 0.08 \text{\mu moles/g tissue}) was observed at 4 hours after treatment (46% of the control level). GSH levels returned to control values (3.22 ± 0.40 \text{\mu moles/g tissue}) at 12 hours after treatment.
In the dose-response studies (Figure 2), significant depletion of GSH in the gastric mucosa (1.79 ± 0.16 μmoles/g tissue; 58% of control level) was observed at 2 hours following a single oral dose (38 mg/kg) of CAN. The data in Figure 2 indicate that increasing CAN doses (76 mg/kg) induced further depletion of GSH contents at 2 hours after administration (1.20 ± 0.24 μmoles/g tissue; 38% of the control).

Effect of Oral Administration of CAN on the Integrity of Gastric DNA

To investigate the effect of administration of a single oral dose (38 mg/kg) of CAN on DNA, total genomic DNA from gastric mucosal tissues of control and CAN-treated animals were isolated and analyzed by agarose gel electrophoresis. Damage in gastric mucosal DNA was assessed by the detection of small molecular weight fragments of genomic DNA molecules (Figure 3). DNA damage was electrophoretically detected at 6 and 12 hours following the administration of a single oral dose of CAN. Smearing of genomic DNA (lanes 4 and 5) was observed in gastric DNA of treated animals as compared to the DNA of gastric mucosa of control animals (lane 1). Gastric DNA obtained at 24 hours after CAN (38 mg/kg) treatment showed less fragmentation (lane 6) as compared to earlier times. Although equal amounts (1 μg/20 μL) of DNA samples were electrophoresed in all lanes, the fluorescence of ethidium bromide staining was stronger in DNA extracted from animals treated with CAN and killed at 6 or at 12 hours later. We recently (14) described that cyanomethylation of DNA results in the formation of highly fluorescent product. Similar reaction may have occurred in vivo in animals that received CAN. These results demonstrate that a single oral dose of CAN induced a time dependent DNA damage as illustrated by DNA fragmentation pattern. However, extensive DNA fragmentation has slightly diminished at 24 hours after treatment.

Formation 8-OHdG in Gastric DNA

The possibility of DNA oxidation as a mechanism of CAN-induced DNA damage was investigated. 8-Hydroxylation of guanine bases in DNA of rat pyloric mucosa was determined as a marker for such oxidative damage following various doses of CAN.

Following a single oral dose (38 mg/kg) of CAN, elevated levels of 8-OHdG were detected at 2 and 4 hours after treatment (131 and 134% of control, respectively). At 6 hours after treatment, a significant elevation in gastric 8-OHdG to 146% [9.59 ± 0.60 (8-OHdG/10^5dG)] of control values [6.55 ± 0.71 (8-OHdG/10^5dG)] was observed (Figure 4). Values of
FIGURE 5. Dose response of 8-OHdG levels in gastric DNA of rats treated orally with different doses of CAN as indicated and killed at 2 hours after treatment. Control animals received only solvent. Values are expressed as mean ± SE (N = 3–4). *Significantly different from control (P = 0.0145).

8-OHdG in gastric mucosa at 12 and 24 hours after treatment with this dose were still high [8.51 ± 1.12 and 7.73 ± 0.37 (8-OHdG/10^5dG)] but were not significantly different from control (Figure 4).

The data in Figure 5 indicate that there is a dose-response relationship in CAN-induced 8-OHdG formation. The administration of a single oral dose of 38 mg CAN/kg elevated the levels of 8-OHdG in gastric DNA [8.57 ± 0.83 (8-OHdG/10^5dG)] to 131% of control level at 2 hours following treatment. Increasing the dose to 76 mg/kg, caused a significant increase in gastric 8-OHdG concentrations to 177% [11.59 ± 1.30 (8-OHdG/10^5dG)] of control values at 2 hours following CAN administration (Figure 5). These data demonstrate that a single high dose of CAN significantly increased 8-OHdG concentrations in gastric DNA at an early time after administration.

DISCUSSION

The current in vitro study indicates that gastric mucosal cells are capable of metabolizing CAN to cyanide. Cyanide is known to induce hypoxia (43) and inhibit mitochondrial respiration (17). Cyanide induces lipid peroxidation, depletion of cellular GSH, inhibition of oxidative metabolism, and accumulation of calcium in tissues (44–48). Furthermore, the data also demonstrate that CAN or its metabolites cause reduction of ATP levels in gastric mucosal cells. Depletion of ATP is the initial event that leads to decrease in oxidative phosphorylation and formation of reactive oxygen metabolites (49).

The relationship between CAN-induced GSH depletion and the magnitude of oxidative damage to gastric DNA was investigated. A single oral dose of CAN induced a significant decrease in GSH levels in gastric mucosal tissues. Martensson et al. (16) reported that gastrointestinal epithelial cells are dependent on GSH for its normal function. Depletion of GSH in these tissues leads to marked cellular degradation and damage. Moreover, depletion of GSH by buthionine sulfoximine, a specific inhibitor for γ-glutamyl cysteine synthetase, markedly decreased hepatic and gastric GSH levels and increased gastric injury. Thus extracellular GSH plays an additional role in the protection of gastric tissues when animals are challenged with various types of stress (50). Martensson et al. (23) described a model for oxidative stress in which GSH depletion renders normal and endogenous physiological formation of reactive metabolites largely unopposed, and therefore, oxidative tissue damage may occur.

Smearing of gastric mucosal DNA observed in agarose gel electrophoresis (Figure 3) suggests DNA degradation. Electrophoretic assessment of DNA fragmentation is an unquestionable marker of DNA strand breaks resulting from chemical or physical injury to the cell. Furthermore, Kwon et al. (51) showed that electrophoretic smearing of DNA is a good marker for DNA damage induced by reactive oxygen species. Our results indicate that a single oral dose of CAN triggers gastric DNA fragmentation at 6 and 12 hours following treatment (Figure 3). This pattern of DNA fragmentation correlates with the observed GSH depletion. At 24 hours after treatment, the fragmentation pattern shown by electrophoresis was decreased when GSH levels were fully rebound.

A single oral dose of CAN caused a significant elevation of 8-OHdG (46% above the control values) at 6 hours after administration. GSH depletion, which was maximum at 4 hours after CAN treatment, seems to be succeeded by the elevated levels of 8-OHdG at 6 hours. The data indicate that 8-OHdG peak comes after GSH depletion. The latter phenomena may be ascribed to accumulation of reactive oxygen species in the cells following GSH depletion (Fig. 6). This finding illustrates the protective role of GSH in gastric tissues against oxidative damage to DNA. High dose of CAN caused further depletion of GSH levels, followed by high levels of 8-OHdG in gastric tissues at early times (2 h) after treatment.

Increasing evidence suggests that ischemia-associated gastric injury is related to the production of oxygen-derived free radicals during reperfusion (52,53). Oxidative DNA damage as indicated by the formation of 8-OHdG plays a significant role in gastric carcinogenesis (29). Furthermore, levels of 8-OHdG are found to be reliable markers of DNA damage in various hu-
man diseases (54). CAN is metabolized to cyanide in the gastric mucosal cells (Fig. 6). Cyanide incites reduced oxygen utilization, thus leading to excessive formation of oxygen free radicals. The latter may cause oxidative stress in gastric mucosal cells, as indicated by DNA fragmentation and increasing levels of 8-OHdG.

In conclusion, many exogenous and endogenous agents can disturb the delicate balance between reactive oxygen species and antioxidant mechanisms such as GSH homeostasis. The combined cyanide release and GSH depletion induced by CAN or its metabolites, as shown in Figure 6, may disturb such balance and lead to oxidative damage of gastric DNA. The increase in gastric 8-OHdG levels induced by CAN is significant when compared to DNA damage induced by other common carcinogens (26,28).

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