

BROMOTRICHLOROMETHANE (CBrCl₃) treatment is a model for studies on molecular mechanisms of haloalkane toxicity with some advantages compared with CCl₄ treatment. The formation of 4-hydroxynonenal and similar aldehydic products of lipid peroxidation, which play a role as mediators of inflammatory processes, was clearly demonstrated in rat hepatocytes treated with CBrCl₃. It may be assumed that haloalkane toxicity is connected with the biological effects of those inflammation mediatory aldehydic compounds.

Key words: Aldehydes, Bromotrichloromethane, Haloalkanes, Hepatocytes, 4-Hydroxynonenal, Lipid peroxidation

Formation of 4-hydroxynonenal and further aldehydic mediators of inflammation during bromotrichloromethane treatment of rat liver cells

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Introduction

Although pathophysiological and biochemical changes due to haloalkane intoxication have been investigated intensively by many laboratories, increasing exposure to toxic pollutants in the workplace and in the environment requires the continuation of studies on the molecular basis of haloalkane toxicity. Haloalkanes are also of importance in clinical practice because of the use of halothane and other halogenated compounds as inhalation anaesthetics, and a number of clinical cases of CCl₄ poisoning. Additionally 1,2-dibromomethane has been used as a fruit and grain fumigant.

Haloalkanes induce serious inflammatory reactions. Several reports exist on biochemical changes in plasma during halothane treatment characterizing these inflammatory reactions,^{1,2} and on hepatitis and cirrhosis after halothane or CCl₄ intoxication.³ The cytotoxic mechanisms of haloalkanes have been studied mostly in cell suspensions, organs or whole animals treated with CCl₄. CBrCl₃ was also used several times for investigations on haloalkane toxicity. CCl₄ is metabolized in a cytochrome P-450 dependent reaction which leads to the formation of the [•]CCl₃ radical. The [•]CCl₃ radical rapidly interacts with membrane lipids.⁴⁻⁷ The [•]CCl₃ induced lipid peroxidation following CCl₄ treatment is con-

siderably lower than that induced by CBrCl₃ treatment.⁸ It is known that the C–Cl cleavage needs more energy (68 kcal/mol) than the cleavage of the C–Br bond (49 kcal/mol).⁹ Koch *et al.*⁸ investigated comparatively the capacity of CCl₄ and CBrCl₃ to induce lipid peroxidation. *In vitro*, CBrCl₃ induced lipid peroxidation was about 100 times more effective than the CCl₄ induced lipid peroxidation,⁸ and *in vivo* was three times more effective. That favours the CBrCl₃ model for studies on cytotoxic effects of haloalkanes in comparison with the CCl₄ model.

The most common parameter for the measurement of lipid peroxidation and its products is the generation of malondialdehyde (MDA) in exposed systems. However, the various procedures of MDA determination produce different results.¹⁰ Furthermore, those methods are rather unspecific for the free metabolite MDA.¹¹ The determination of other aldehydes—e.g. 4-hydroxynonenal (HNE), as the main representative of 4-hydroxyalkenals—is a more specific and sensitive method for the estimation of lipid peroxidation. It is known that 4-hydroxynonenal and some other aldehydic products of lipid peroxidation are much more effective as inflammatory agents, e.g. as chemo-attractive substances, than MDA.¹² Poli *et al.* determined the formation of various aldehydic products of lipid peroxidation during CCl₄

treatment in hepatocyte suspensions.¹³ MDA formation was measured in CBrCl₃ treated cells too,^{4,5,14} but there are no data on the formation of 4-hydroxyalkenals and further aldehydic products of lipid peroxidation except MDA in CBrCl₃ treated cells, organs or animals. This paper deals with the determination of such highly reactive mediators of inflammation in the CBrCl₃ intoxication model.

Materials and Methods

Chemicals: Aldehyde standards (dinitrophenylhydrazones) were prepared in the laboratory of Prof. H. Esterbauer, Karl-Franzens University Graz, Austria. The solvents *n*-hexane, acetonitrile, dichloromethane, methanol and benzene, and the TLC plates (silica gel 60, 0.2 mm thickness) were from Merck (Darmstadt, Germany). 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Union Chimique (Brussels, Belgium). DNPH was dissolved in 5 ml of 1 M hydrochloric acid, extracted with 3 × 5 ml of *n*-hexane, then adjusted to 1.8 mM solution with 1 M hydrochloric acid by means of absorbance measurement at 378 nm. Collagenase (*Clostridium histolyticum*) was from Boehringer (Mannheim, Germany). All other reagents were purchased from Sigma Chemie GmbH (Deisenhofen, Germany). Thiobarbituric acid and malondialdehyde diethylacetal were obtained from Aldrich Chemie GmbH (Steinheim, Germany).

Cell preparation: Male Wistar H-strain rats with a body weight of about 230 g were used. Hepatocytes were prepared according to the method given in Berry and Friend¹⁵ with modifications given in Vincent¹⁶. Starved rats (24 h) were anaesthetized by an intraperitoneal injection pentobarbital (30 mg/kg body wt.). As perfusion medium a Krebs–Henseleit bicarbonate buffer gassed with O₂:CO₂ (19:1) was used.

Cell incubation: The cell viability was determined by cell staining with trypan blue. Only hepatocyte suspensions with more than 85% of viable cells excluding trypan blue were used for experiments. The cell suspensions were adjusted to a cytocrit of 2% (v/v) (5.2 × 10⁶ cells/ml) with Krebs–Henseleit buffer as the incubation medium. The cells were incubated at 37°C under continuous gyratory shaking. CBrCl₃ dissolved in ethanol was added in a final concentration of 1 mM. The controls were treated with the same amount of ethanol (1% final concentration). The aldehydic formation in hepatocyte suspensions induced by 1 mM CBrCl₃ was compared with aldehyde formation in hepatocyte suspensions which were incubated in presence of 1 mM CCl₄ under the same conditions of preparation, incubation, extraction and analysis.¹³

Determination of 4-hydroxynonenal and further aldehydes: The detection of aldehydes was performed according to Esterbauer *et al.*¹⁷ and Poli *et al.*¹³

Derivatization of aldehydes with DNPH: 2.5 ml of the cell suspension reacted with 2.5 ml of DNPH solution (1.8 mM in 1 N HCl) in the presence of butylated hydroxytoluene (BHT) (final concentration 10 mM) for 2 h in the dark. Thereafter, the sample was kept on ice for 1 h in the dark, then extracted three times with 8 ml of dichloromethane and centrifuged at 900 × g. The supernatant was evaporated on a rotary evaporator to dryness at 33°C. The residue, dissolved in 1 ml of dichloromethane, was transferred into small vials for spotting on TLC plates.

Thin-layer chromatography: The TLC plates were developed with dichloromethane in comparison with known standards. By this method according to details given in Esterbauer *et al.*¹⁷ the dinitrophenylhydrazones of carbonyl compounds were separated into three zones. Zone I contained the 4-hydroxyalkenals. Zone I and Zone III were scraped off, each extracted three times with 10 ml of methanol and evaporated to dryness. The residue was dissolved in 1 ml of methanol.

HPLC equipment and chromatographic conditions: A HPLC system from Perkin Elmer was used consisting of a M410 pump system, a LC 95 variable wavelength detector, a LCI-100 integrator and a Rheodyne injector. For some determinations a Photodiode-Array-Detector (Hewlett–Packard) was used additionally.¹⁸ The eluent for the isocratic separation was methanol–water (4:1, v/v), at a flow rate of 1 ml/min. A Nucleosil 5C18 column (Macherey, Nagel & Co., Düren, Germany, 250 × 4.0 mm I.D.) was used, with a 20 × 4.0 mm I.D. pre-column. Peak identification was performed by comparison of the retention times of peaks of biological extracts and of standard extracts, and also by the coelution of biological extracts with the reference compound and by comparison of the spectra of the analytes. Quantification of HNE was achieved by separating HNE–DNPH standard solutions of different concentrations. Quantification of further aldehydes was also performed using different concentrations of their dinitrophenylhydrazone derivatives.

Recovery: The recovery was determined using a HNE standard solution (10 μM) and amounted to 32 ± 5% (mean ± S.D.). The value of HNE recovery was highly reproducible. Recoveries of other carbonyls were quantified in an analogous manner. All recoveries were between 25 and 40%. The values of HNE and other aldehydes were corrected to 100%.

Determination of malondialdehyde (MDA): MDA was measured according to Wong *et al.*¹⁹ A 200 μ l sample of cell suspension was mixed with 0.75 ml phosphoric acid (0.44 mol/l), 0.25 ml thiobarbituric acid (42 mmol/l) and 0.3 ml water and boiled for 60 min. The reaction was stopped by cooling the samples in an ice bath. Immediately before HPLC analysis an equal volume of 1 M NaOH was added followed by centrifugation. The HPLC system consisted of a Waters 510 pump, a Shimadzu fluorescence detector RF-530 (525/550 nm) and a C-R6A integrator. The column was a Supelcosil 150 \times 4 mm LC-18-S (5 μ M). As eluent, a 50 mM potassium phosphate buffer solution (pH 6.8) with 40% methanol was used.

Results

In this study of HNE, MDA and further aldehydic products of lipid peroxidation could be detected and quantitatively evaluated in the samples of hepatocyte suspensions. There were no differences between HNE levels of controls and CBrCl₃ treated cells at time zero of the experiments (Fig. 1). The HNE concentration of control suspension was constant during 1 hr of incubation. The HNE level was about 200 nmol/l suspension (0.2 μ M). In the case of CBrCl₃ treatment, 5 min after the addition of haloalkane to the cell suspension the HNE level was markedly increased. The HNE value at this time point was about 600 nmol/l cell suspension. This three-fold increase in the HNE level was maintained throughout the whole experiment. There was even a trend for a further slight increase between 45 and 60 min after CBrCl₃ addition.

Figure 2 shows the changes of MDA concentration in CBrCl₃ treated hepatocyte suspensions. The level of MDA at time zero was about 800 nmol/l cell suspension (0.8 μ M). There was a slight decrease of MDA level in control suspensions during the first 15 min of incubation. Therefore, the

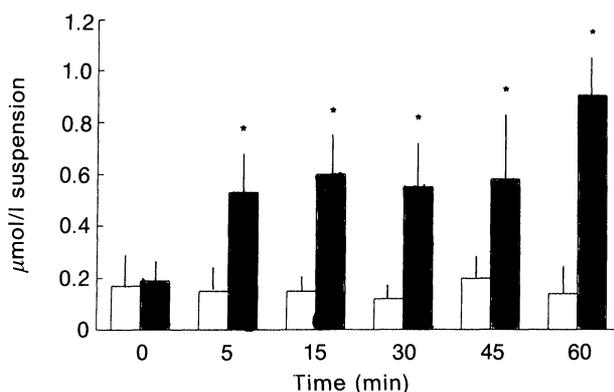


FIG. 1 Concentration of 4-hydroxynonenal in hepatocyte suspensions after bromotrichloromethane treatment. Symbols are: □ Control (addition of ethanol), ■ bromotrichloromethane treatment (1 mM). Values are given as mean \pm S.D. (* p < 0.05 as compared with controls).

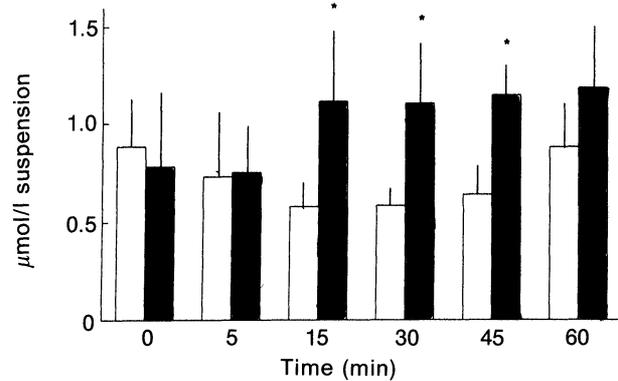


FIG. 2 Concentration of thiobarbituric acid—reactive substances in hepatocyte suspensions after bromotrichloromethane treatment. Symbols are: □ Control (addition of ethanol), ■ bromotrichloromethane treatment (1 mM). Values are given as mean \pm S.D. (* p < 0.05 as compared with controls).

Table 1. Levels of aldehydic products of lipid peroxidation in hepatocyte suspensions treated 60 min with 1 mM CBrCl₃. Values are given as nmol/l cell suspension; mean \pm S.D. (six experiments)

Aldehyde	0 min	60 min
MDA	782 \pm 385	1180 \pm 355
HNE	190 \pm 70	900 \pm 160
Hexadienal	2.4 \pm 1.0	6.3 \pm 1.8
Heptadienal	0.6 \pm 0.6	6.0 \pm 1.0
Nonadienal	7.5 \pm 2.6	11.7 \pm 2.2
Decadienal	13.2 \pm 4.0	24.2 \pm 4.4
Pentanal	9.3 \pm 1.7	27.1 \pm 2.9
Heptanal	26.0 \pm 5.4	40.7 \pm 4.0

control values of MDA level were in the range of 0.5 to 0.8 μ M. CBrCl₃ treatment increased the MDA concentration within 15 min (about twice the initial value). Table 1 represents data on the further aldehydic products of lipid peroxidation during 60 min of CBrCl₃ treatment of hepatocyte suspensions. It was possible to detect low amounts of such aldehydic compounds in CBrCl₃ treated cells. In Fig. 3 the accumulation of MDA, HNE, pentanal and heptanal in CCl₄ and in CBrCl₃ treated hepatocyte suspensions is compared.

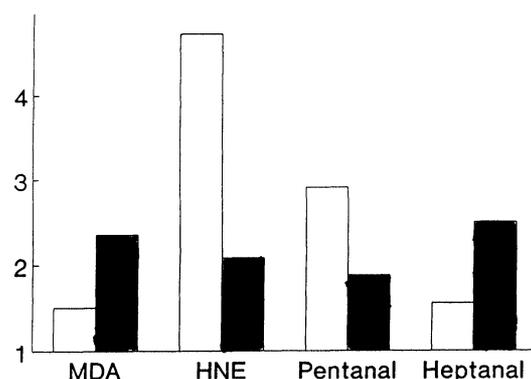


FIG. 3 Concentration ratios of aldehydes after 60 min of incubation with □ CBrCl₃ and ■ CCl₄ (see Reference 13) compared with the initial concentration in the same suspension.

Discussion

Pathophysiological role of aldehydic products of lipid peroxidation: There is increasing evidence that aldehydes generated during the process of lipid peroxidation are causally involved in some of the pathophysiological effects associated with oxidative stress in cells and tissues.²⁰ Unlike reactive free radicals, aldehydes are rather long-lived and can therefore diffuse from the site of their origin, i.e. membranes, and reach and attack targets intracellularly or extracellularly which are distant from the initial free radical event. HNE and similar aldehydic products of lipid peroxidation exhibit a wide spectrum of cytotoxic effects as well as mutagenic and genotoxic effects.²⁰⁻²³

Since HNE, and other aldehydes, are formed during the acute phase of inflammation they may play a role as mediators in the inflammatory process.²⁰ HNE effects produced even at HNE concentrations of 0.1 μM or less include stimulation of chemotactic oriented migration of neutrophils,^{12,24,25} modulation of adenylate cyclase activity, weak stimulation of guanylate cyclase²⁶ and stimulation of phospholipase C.²⁷ Phospholipase C is important for signal transduction by G-proteins. Stimulation of phospholipase C leads to increased hydrolysis of phosphatidyl-4,5-bisphosphate with concomitant formation of diacylglycerol and inositol triphosphate. Diacylglycerol acts synergistically with inositol triphosphate in the calcium dependent activation of protein kinase C. Inositol triphosphate opens calcium channels, whereas diacylglycerol increases the calcium affinity of protein kinase C. Protein kinase C plays an important role in the control of cell division, cell proliferation and other cellular functions. Phospholipase C stimulation may also be responsible for the chemotactic effects of 4-hydroxynonenal since it is known that the chemotactic signal of f-met peptides also results in an activation of phospholipase.²⁸

4-Hydroxyalkenals induce the oriented migration and morphological polarization of neutrophils in a concentration range of 10^{-6} to 10^{-12} M depending on the chain length of the aldehyde. The most effective aldehyde was not HNE but 4-hydroxyoctenal with a maximum chemotactic activity at 10^{-11} M. For HNE the effective doses for chemotactic effects and for activation of phospholipase C are between 10^{-6} and 10^{-9} M.²⁵

Cytotoxic haloalkane effects depend on the formation and action of free radicals initiating lipid peroxidation.^{4,5,29} The formation of HNE and similar aldehydic products of lipid peroxidation in hepatocytes treated with CCl_4 was published by Poli *et al.*¹³ In the present study the formation of such compounds could also be demonstrated for CBrCl_3 .

Critical aspects of methodological approach: For the determination of long chain aldehydes a number of methods, such as the dinitrophenylhydrazine method, the direct determination by HPLC, the cyclohexadione method and the determination by GC-MS, are described in Esterbauer and Zollner³⁰. The determination of a wide spectrum of aliphatic aldehydes is only possible, using the dinitrophenylhydrazine and the cyclohexadione method, with comparable results in recovery and reproducibility. The literature results for reproducibility and low recovery (about 40%) are in accordance with those achieved in this study.^{30,31} The determination of the relatively low recoveries for each aldehyde allows the determination of the real biological steady state concentration in the cell suspension. Nevertheless, the low recovery and therefore a certain inaccuracy of methods which were available for aldehyde analysis modify the quantitative aspect of those measurements.

Comparison of formation of aldehydes as mediators of inflammation in CCl_4 and CBrCl_3 toxicity: The initial levels of MDA and of HNE at zero time were in the same range as concentrations reported by other authors in hepatocyte suspensions.¹³ In contrast to CCl_4 treated cells the aldehyde levels increased very rapidly, i.e. within the first minutes following CBrCl_3 addition to the hepatocytes. However, the increases of MDA and HNE levels in hepatocyte suspensions treated with CBrCl_3 were in the same range as in those hepatocyte suspensions treated with equimolar concentrations of CCl_4 .¹³ The increase of some aldehydic products of lipid peroxidation within 1 h of treatment with CCl_4 or CBrCl_3 in comparison to initial levels is demonstrated in Fig. 3. The most interesting point of this comparison seems to be the excessive increase of HNE as a very cytotoxic and chemotactic compound at CBrCl_3 loading compared with CCl_4 loading.

The generation of aldehydic products of lipid peroxidation other than MDA and HNE could be demonstrated for CBrCl_3 incubations (Table 1) but at markedly lower concentrations than those for MDA and HNE. Nevertheless, the amounts of these aldehydes and of HNE which were produced even during short-term experiments are high enough for the initiation of chemotactic activity towards neutrophils.

It should be mentioned that the determined aldehyde concentrations represent, of course, the steady state concentrations, resulting from a continuous formation of the aldehydes and a consumption of these compounds by the hepatocytes. The HNE metabolism—including that in hepatocytes—was investigated in several studies.^{13,32-35} In general the maximal capacity of tissues to

metabolize aldehydes, especially HNE, is in the order of several micromoles per gram wet weight per minute, which is about six orders higher than the accumulation within the first minutes after haloalkane treatment.

The specific pattern of aldehydes formed by CBrCl_3 and CCl_4 is different, whereas the overall increase in MDA and HNE is comparable for both compounds. The differences in lifetime, reactivity and 'consumption' rate of aldehydes may implicate differences in the pattern of cell and tissue damage if a different aldehyde pattern occurs. There is the possibility that in CBrCl_3 induced liver damage 4-hydroxynonenal and similar aldehydic products of lipid peroxidation are involved in cell damage and act as mediators of inflammatory processes. The relation between formation and 'consumption' of aldehydes should be investigated in further experiments.

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