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N-Nitrosocarbofuran, but not Carbofuran, induces apoptosis and cell cycle arrest in CHL cells

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Abstract

Carbofuran (CF) is one of the most widely used carbamate pesticides in the world applied for insect and nematode control. Due to its widespread use in agriculture and households, contamination of food, water, and air has become serious, and consequently adverse health effects are inevitable in humans, animals, wildlife and fish. It has been reported that CF alone or in combination with other carbamate insecticides influences the level of reproductive and metabolic hormones such as thyroxine and corticosterone, and results in impairment of endocrine, immune and behavioral functions. In this study, we evaluated the effects of CF and its metabolite, the *N*-nitroso derivative *N*-nitrosocarbofuran (NOCF), on genotoxicity, cell growth, cell cycle and apoptosis of Chinese hamster lung fibroblast (CHL) cells. NOCF, but not CF, induced genotoxicity determined by Ames test. NOCF inhibited the growth of Chinese hamster lung fibroblast (CHL) cells with an IC_{50} of 12.8 μM. NOCF induced apoptosis of CHL cells, which was demonstrated by morphological changes, DNA fragmentation and flow cytometric analysis. Treatment of CHL cells with NOCF induced significant G_2/M cell cycle arrest. Caspase-3, an executioner of apoptosis was also activated by the treatment of CHL cells with NOCF. These results suggest that NOCF, that is an important metabolite of CF, leads to the induction of cell cycle arrest and apoptosis in CHL cells.

Keywords: *N*-methylcarbamate; Carbofuran; *N*-Nitrosocarbofuran; O^6-Methylguanine; Apoptosis; Cell cycle arrest

Abbreviations: Ac-DEVD-*p*NA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; CF, carbofuran; CHL, Chinese hamster lung fibroblast; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MTT, 3-(4-,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; O6MeG, O6-methylguanine; PBS, phosphate buffered saline; PI, propidium iodide; TUNEL, terminal deoxynucleotydyl transferase-mediated dUTP nick-end labeling.

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1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate; CF) is one of the most widely used *N*-methylcarbamate esters applied for insect and nematode control. Acute toxic effects induced by *N*-methylcarbamate pesticides are caused by the inhibition of acetylcholine esterase in the nervous tissue; the inhibition is labile, of short duration, and reversible compared with that induced by organophosphate compounds (Gupta 1994; Maroni et al., 2000) Due to its widespread use in agriculture and household, contamination of food, water and air has become serious, and consequently adverse health effects are inevitable in humans, animals, wildlife and fish. A growing body of epidemiological studies suggests that long-term exposure to relatively safe pesticides, including CF, may be associated with increased risk of gastrointestinal, neurological and cardiac dysfunction as well as retinal degeneration (Cole et al., 1998; Peter and Cherian 2000; Kamel et al., 2000). CF has been reported to be detected repeatedly in drinking water supplies with a typical concentration range between 1.0 and 5.0 μg/l (Hallberg, 1987). Therefore, the contamination of human environment with CF can easily occur and there is an increasing risk of a long-term low dose exposure to this pesticide. Moreover, it has been reported that CF alone or in combination with other carbamate insecticides influences the level of reproductive and metabolic hormone, resulting in impairment of endocrine, immune and behavioral functions (Cranmer et al., 1978; Rawlings et al., 1998).

Carbamate pesticides are readily converted to *N*-nitroso metabolites in the presence of nitrites or nitrogen oxides in the stomach under acidic conditions or in the colon by intestinal bacteria (Elespuru and Lijinsky, 1973; Regan et al., 1976; Hughes et al., 2001). It is reported that 0.5–2% of the carbamate dose were isolated as the nitroso derivative from the guinea pig stomach (Rickard and Dorough, 1984). The nitroso derivatives of carbamate pesticides lose the cholinesterase inhibiting properties of the parent compounds and are less toxic to mammals. However, they induce sister chromatid exchange, chromosomal aberration, micronucleus formation and aneuploidy in vitro and in vivo (Gonzalez Cid et al., 1990; Soderpalm-Berndes and Onfelt, 1988; Chauhan et al., 2000). Like most of the *N*-nitrosoamide compounds, *N*-nitrosocarbamates are potent direct acting mutagens and carcinogens. *N*-Nitrosocarbofuran (NOCF) is mutagenic (Nelson et al., 1981) and induces DNA single strand breaks in human skin fibroblasts (Blevins et al., 1977).

Apoptosis is a highly regulated process by which an organism eliminates unwanted cells without eliciting an inflammatory response. Some abnormal physiological and chemical injuries, therefore, induce apoptosis (Pallardy et al., 1999). Apoptosis is also triggered in response to various forms of stress and DNA damages (Smith and Fornace 1996; Park et al., 1998). Apoptosis induced by mutagenic or genotoxic carcinogens is vital because it eliminates cells harboring mutagenic DNA damage from the body. In fact, human cancers contain cells that have been potentially compromised in their ability to undergo apoptosis (Hollstein et al., 1991). Only a limited number of papers deal with the genotoxic mechanism of NOCF. Wang et al. (1998a,b) reported recently that *N*-nitrosation greatly increased the cytotoxicity and mutagenicity of *N*-methylcarbamates at the *hprt* locus in Chinese hamster V79 cells. They showed that the major mutational change is a transition from GC to AT, which is similar to the results reported for *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). We report in the present study on the cellular response in Chinese hamster lung fibroblast (CHL) treated with NOCF in terms of the induction of cell cycle arrest and apoptosis.

2. Materials and methods

2.1. Chemicals

CF was purchased from Aldrich Chemical Co (Milwaukee, WI) and NOCF was synthesized by nitrosating CF in an acidic condition in the presence of sodium nitrate (Fig. 1) (Blevins et al., 1977). Stock solutions (100 mM) of CF and NOCF were prepared by dissolving the compounds in dimethyl sulfoxide.

2.2. Cell culture

CHL cells were maintained in minimum essential medium supplemented with 5% fetal bovine serum (GIBCO BRL, Grand Island, NY). Cells were harvested using 0.2% trypsin and seeded onto 100 mm culture dishes. The cells were then allowed to grow at 37 °C in a 5% CO_2-95% air humidified incubator.

2.3. Ames test

The preincubation method of Maron and Ames (1983) was carried out using *S. typhimurium* TA100 in the presence and absence of S9 mixture. S9 fraction was prepared from the pooled livers of Aroclor 1254-induced Sprague–Dawley rats.

2.4. Cell proliferation assay

Cell proliferation was determined by the 3-(4-, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. CHL cells were seeded into a 96-well plate at a concentration of 3×10^4 cells/ml in 100 μl of medium and left overnight to adhere. The chemicals in 100 μl volumes were added and incubated for different time periods as indicated in the figure legend. General viability of cultured cell was determined by reduction of MTT to formazan following 96 h of incubation (Monks et al., 1991). The concentration required for 50% inhibition of the growth (IC_{50}) was determined by non-linear regression analysis using the GraphPad PRISM™ statistics software package (Ver. 2.0; San Diego, CA).

2.5. Morphological analysis and TUNEL assay

Cells were treated with NOCF for 48 h, washed gently three times with phosphate buffered saline (PBS) and fixed with paraformaldehyde for 30 min. The cells were washed with PBS and then stained with propidium iodide (PI) solution (50 μg/ml of PI with 100 μg/ml of RNase). The morphology of the cells was examined using the Leitz phase-contrast microscope or Olympus Fluoview laser scanning confocal microscope. Apoptosis was also detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method using the Boehringer in situ death detection kit according to the instruction of the supplier (Mannheim, Germany).

2.6. DNA fragmentation analysis

The cells grown at a density of 2×10^6 cells/ml in 100 mm culture dishes were treated with appropriate concentrations of the chemicals as described in the figure legends. Genomic DNA was prepared with Wizard Genomic DNA Purification Kit (Promega, Madison, WI). DNA was precipitated with isopropanol, separated in 1.5% agarose gel and visualized by UV illumination after ethidium bromide staining.

2.7. Flow cytometry and cell cycle analysis

Exponentially growing cells were diluted and 2×10^6 cells were seeded in 10 ml of culture medium in 100 mm culture dishes. They were

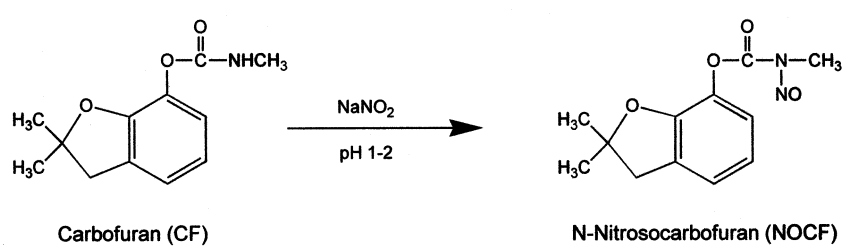

Fig. 1. The formation of NOCF from CF in acidic condition.

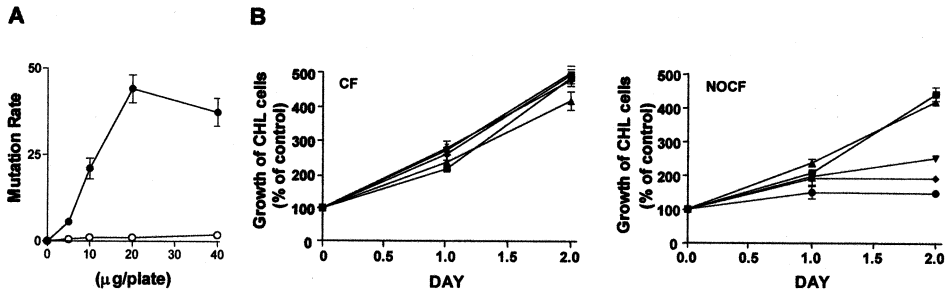

Fig. 2. Mutagenicity and antiproliferative effect of CF and NOCF. Genotoxicity of CF (○) and NOCF (●) was determined by Ames test in the absence of metabolic activation system (A). Mutation rate was calculated by dividing the mean number of test reverent colonies by that of spontaneous reverent colonies. Cells were incubated with CF or NOCF (■, control; ▲, 1 μM; ▼, 10 μM; ◆, 30 μM; ●, 50 μM) and the time-course of proliferation was determined by MTT assay as described in Section 2 (B). Each point represents the mean and standard error from three experiments.

exposed to CF or NOCF for 48 h and centrifuged at 800 × *g* for 5 min. The pellets were mixed with 1:1 (v/v) mixture of PBS and 0.2 M Na_2HPO_4-0.1 M citric acid (pH 7.5) and fixed with ice-cold ethanol at 4 °C for 1 h. The cells were washed twice with PBS and resuspended in 1 ml of a staining solution containing 10 μg/ml PI containing 100 μg/ml DNase-free RNase A. The cell suspensions were incubated at room temperature for 1 h and 20 000 cells were analyzed on a FACScalibur flow cytometer (Becton–Dickinson)

2.8. Caspase-3 activity

Substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; Ac-DEVD-*p*NA) was added to the cell lysates in assay buffer (50 mM HEPES; 100 mM NaCl; 0.1% CHAPS; 10 mM dithiothreitol; 1 mM EDTA; 10% glycerol; pH 7.4) and incubated for 1 h at 37 °C. The cleavage of the peptide substrate was monitored at 405 nm.

3. Results

3.1. Genotoxicity and cytotoxicity of CF and NOCF

We have tested the genotoxicity of CF and NOCF using the Ames test with *S. typhimurium* TA100. NOCF was mutagenic in the absence of metabolic activation system, while CF was not. The mutagenicity of NOCF was increased in a dose dependent manner up to a concentration of 20 μg per plate, which is in accordance with the previous results (Fig. 2, A). Neither CF nor NOCF was mutagenic in the presence of metabolic activation system (data not shown). The effects of CF and NOCF on the proliferation of CHL cells were tested. Cells were treated with the compounds for 48 h and the number of viable cells was determined by MTT assay. The treatment of CHL cells with NOCF significantly inhibited the growth of the cells in a dose dependent manner. The IC_{50} at 48 h of incubation was 12.8 μM. On the other hand, CF did not influence the growth rate of CHL cells at concentrations between 1 and 50 μM (Fig. 2, B).

3.2. Induction of apoptosis by CF and NOCF in CHL cells

During the course of the cytotoxicity experiment we observed that NOCF induced morphological changes that are characteristic of apoptosis. Although untreated cells exhibited typical adherent and well-spread morphology, cells exposed to NOCF frequently displayed round shape and condensation of chromatin. To clarify the mode of cell death induced by NOCF, DNA fragmentation analysis, confocal microscopy of nuclear morphology and TUNEL staining were performed. Fig. 3A, depicts the results of the agarose gel electrophoresis of cellular DNA showing that NOCF treatment induced an oligosomal DNA ladder. NOCF exposure induced substantial

nuclear condensation, shrinkage and a loss of internal nuclear structure. Staining of apoptotic nuclear buds and fragments was also observed (Fig. 3, B-c). TUNEL staining shows an intense fluorescence in NOCF treated cells (Fig. 3, B-f). Treatment of CF did not alter the morphology and TUNEL staining of the cells (Fig. 3, B-b, B-e).

3.3. Flow cytometry and cell cycle analysis

Flow cytometric analysis was performed to quantify the degree of apoptosis by NOCF and to determine the effect of NOCF on the cell cycle distribution of the CHL cells. NOCF, but not CF, resulted in the progressive generation of cells with hypodiploid DNA content (Fig. 4A) that is characteristic of apoptosis. Quantitation of the apoptotic cells shows a dose-dependent response to NOCF (Fig. 4B), and this result is in good accordance with that of the DNA fragmentation assay by agarose gel electrophoresis. The histogram of the cells treated with 30 μM NOCF shows an increase in the G_2/M phase and a decrease in the G_1 phase. The quantitative analysis of the histograms to determine the percentage of cells in the G_1, S, and G_2/M phases for the control cells and the cells treated with NOCF for 48 h is shown on Fig. 4C. The data clearly indicate that NOCF causes a significant increase in the percentage of G_2/M phase cells and a significant decrease in the percentage of G_1 phase cells, whereas the percentage of S phase remains unchanged.

3.4. Caspase-3 Activity

Caspase-3 activation is a late signal that accomplishes apoptosis in mammalian cells. To determine whether caspase activation is involved in the NOCF-induced cell death, CHL cells were exposed to NOCF and assayed for caspase-3 activity. Treatment of the cells with NOCF activated

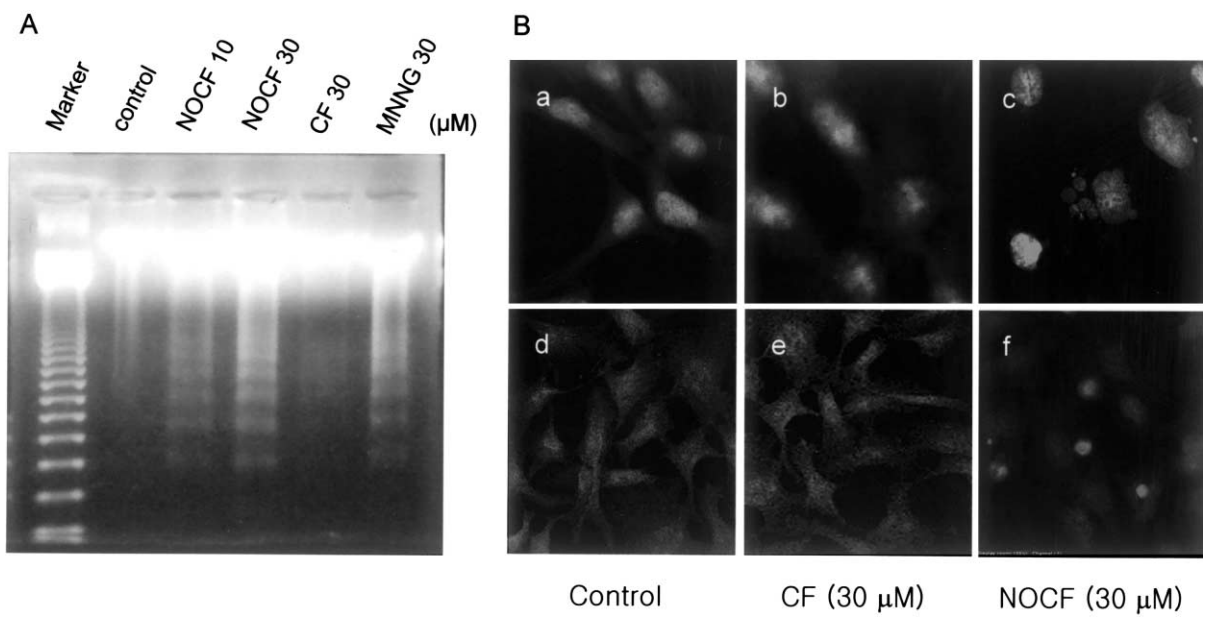

Fig. 3. Morphological analysis and TUNEL assay CHL cells were exposed to CF or NOCF for 48 h. For DNA fragmentation analysis, genomic DNA was extracted, electrophoresed in 1.5% agarose gel and visualized by UV illumination after ethidium bromide staining (A). For morphological analysis, control (B-a), CF (B-b) and NOCF treated cells (B-c) were fixed with paraformaldehyde and stained with PI. NOCF exposure induced substantial nuclear condensation, shrinkage and a loss of internal nuclear structure. Staining of apoptotic nuclear buds and fragments were also observed. For TUNEL analysis, control (B-d), CF (B-e) and NOCF treated cells (B-f) were stained with Boehringer in situ death detection kit according to the supplier's manual. TUNEL staining shows an intense fluorescence in NOCF treated cells.

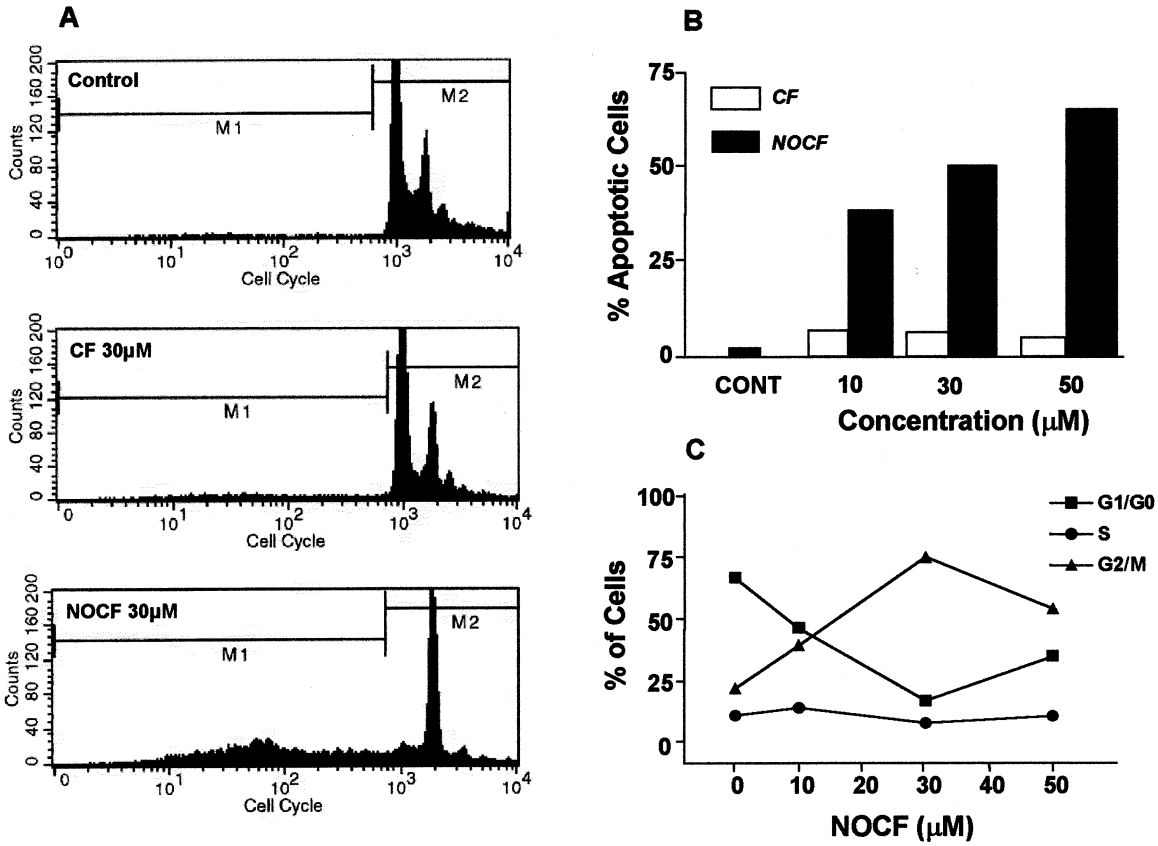

Fig. 4. Flow cytometric analysis of apoptosis and cell cycle distribution. CHL cells were treated with CF or NOCF for 48 h, stained with PI and analyzed on a FACScalibur flow cytometer (A). Percentage of apoptotic cells (B) and dose-dependent changes in CHL cell cycle induced by NOCF (C) is shown at the indicated concentrations. Representative diagrams are presented and the deviation was within the range of 5% in all three independent experiments.

caspase-3 in a dose dependent manner (Fig. 5). The dose-dependent increase in the caspase-3 activity is in good accordance with that in the percentage of apoptotic cells determined by flow cytometric analysis (Fig. 4). CF did not affect the caspase-3 activity at a concentration of 30 μM.

4. Discussion

In the present study, we demonstrated that exposure of CHL cells to NOCF induced cell cycle arrest and apoptotic death of CHL cells. The parent compound CF was neither genotoxic nor cytotoxic in bacterial as well as in mammalian cell systems.

N-Nitroso methylcarbamates induced sister chromatid exchange and micronuclei formation in human lymphocytes (Gonzalez Cid et al., 1990). They are more cytotoxic than their parent compounds, and they induce chromosome aberration and *hprt* gene mutation in V79 cells, and cell transformation in rat tracheal epithelial cells (Wang et al., 1998a,b). *N*-Methylcarbamates do not induce cytotoxicity or genotoxicity, but they inhibit gap-junctional intercellular communication in the cells at doses that produce little cytotoxicity.

It is now generally recognized that total endogenous nitrosation processes contribute significantly to a genotoxic burden for the general population who are not known to have suffered

excessive exposure to any environmental methylating agents (Georgiadis et al., 2000). The observation of a possible protective effect of intake of ascorbic acid and green tea consumption on nitrosation supports the hypothesis that endogenous nitrosation play an important role in the total human exposure to genotoxic methylating agents (Vermeer et al., 1999).

Similarly to the results reported for MNNG (Lukash et al., 1991; Yang et al., 1991), the *hprt* mutation analysis by *N*-nitrosopropoxur revealed that the major mutational change is a transition from GC to AT. Since the GC to AT transition occurs mainly as a result of the O^6MeG adduct formation, Wang et al. (1998b) have postulated that NOCF might produce this type of DNA adduct, if any. O^6MeG is the major mutagenic base derivative formed in DNA on exposure of cells to DNA methylating agents. This modified base mispairs with thymine during DNA replication, and as a result methylating agents are potent mutagens and carcinogens. It is now generally accepted that alkylation-induced cell killing is largely attributable to apoptosis and the O^6MeG acts as a trigger of this toxic response (Kaina et al., 1997; Meikrantz et al., 1998; Ochs and Kaina 2000). Therefore, we postulated that NOCF induces apoptosis, which is triggered by O^6MeG.

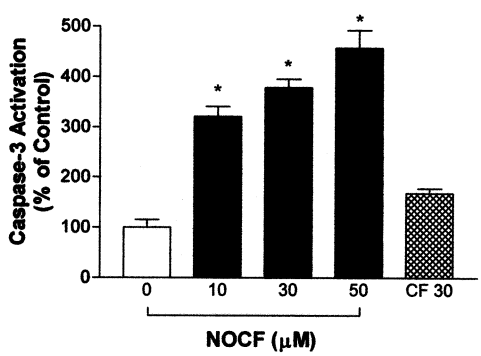

Fig. 5. Activation of caspase-3. Extracts of control cells and cells treated with CF or NOCF were analyzed for caspase-3 activity as a function of concentration. Ac-DEVD-*p*Na was used as the substrate for caspase-3 and the cleavage of the peptide was monitored at 405 nm. The caspase activity of control cells was set to 100% and the relative change in the activity was shown (*$P < 0.01$). Each data represents the mean and standard deviation in three independent experiments.

According to our not yet published results, treatment of calf thymus DNA with NOCF, but not with CF, induced the formation of O^6MeG DNA adduct.

We observed that the treatment of CHL cells with NOCF caused a profound alteration in cell cycle progression. An arrest in G_2/M phase of the cell cycle was induced already with 10 μM of NOCF. Cell cycle arrest is a common response to DNA damaging agents, and such arrest frequently precedes the onset of apoptosis (Meikrantz and Schlegel 1995). DNA damage caused by numerous alkylating agents or ionizing radiation in various cell types has been associated with a G_2 phase delay or arrest (Tobey 1975; Maity et al., 1994; Lin et al., 1999). Activation of p53 can occur in response to a number of cellular stresses, including DNA damage and it controls cell cycle progression and cell survival through apoptosis. However, many commonly used Chinese hamster cell lines, such as Chinese hamster ovarian cell, are defective in p53 function. CHL cells have a spontaneously induced high level of p53 phenotype not inducible by X-irradiation (Hu et al., 1999). Therefore, it seems unlikely that the signal for apoptosis initiated by treatment with NOCF is transmitted by the p53 tumor suppressor protein. We can not, however, exclude the possibility that the p53 protein is mutated by treatment of NOCF, because a mutation at codon 211 of the p53 gene in the Chinese hamster ovarian cell line has been reported to be responsible for the cells losing ability to arrest in the G_1 phase after radiation (Lee et al., 1997; Hu et al., 1999), rather they show G_2/M arrest, as was shown in the NOCF treated cells. Studies are currently under way to address the molecular mechanisms underlying the effects of NOCF on the induction of apoptosis and G_2/M arrest.

Organophosphate or chlorinated hydrocarbon insecticides have been previously reported to induce apoptosis (Carlson et al., 2000; Kannan et al., 2000; Rought et al., 2000). Due to the toxicity and persistence in the environment, pesticides of these classes are not frequently used. *N*-Methylcarbamates, on the other hand, are regarded as relatively safe chemicals because extensive toxicological research demonstrated that these com-

pounds produced only transient, short-term toxicity following acute administration. Our study clearly demonstrates that the *N*-nitroso metabolite of CF is genotoxic and induces cell cycle arrest and apoptosis in CHL cells.

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