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Short communication

N-Nitrosopiperidine and N-Nitrosodibutylamine induce apoptosis in HepG2 cells via the caspase dependent pathway

Almudena García [a], Paloma Morales [a], Joseph Rafter [b], Ana I Haza [a,*]

[a] Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain
[b] Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge University Hospital, NOVUM, S-141 86, Huddinge, Sweden

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Abstract

The human hepatoma cell line (HepG2) exhibited a dose and time-dependent apoptotic response following treatment with N-Nitrosopiperidine (NPIP) and N-Nitrosodibutylamine (NDBA), two recognized human carcinogens. Our results showed a significant apoptotic cell death (95%) after 24 h treatment with NDBA (3.5 mM), whereas it was necessary to use high doses of NPIP (45 mM) to obtain a similar percentage of apoptotic cells (86%). In addition, both extrinsic (caspase-8) and intrinsic pathway (caspase-9) could be implicated in the N-Nitrosamines-induced apoptosis. This study also addresses the role of reactive oxygen species (ROS) as intermediates for apoptosis signaling. A significant increase in ROS levels was observed after NPIP treatment, whereas NDBA did not induce ROS. However, N-acetylcysteine (NAC) did not block NPIP-induced apoptosis. All these findings suggest that NPIP and NDBA induce apoptosis in HepG2 cells via a pathway that involves caspases but not ROS.

Keywords: Apoptosis; Caspases; HepG2 cells; N-Nitrosamines; Reactive oxygen species

1. Introduction

Exposure to N-Nitroso compounds (NOC), which are potential carcinogens, can occur through either ingestion or inhalation of preformed N-Nitrosamines or by ingestion of their precursors (Lijinsky, 1999). Significantly higher amounts of N-Nitrosopiperidine (NPIP) may be formed by nitrosation of piperidine, main principle of pepper, by the nitrite added to the spice mixture (Shenoy and Choughuley, 1992), whereas N-Nitrosodibutylamine (NDBA) is a contaminant in industrial rubber products and rubber toys (Spiegelhalder and Preussmann, 1983). Both NPIP and NDBA are carcinogens in laboratory animals (Gray et al., 1991; Magee and Barnes, 1967) and possible causative agents in human cancer (IARC, 1978).

Apoptosis is characterized by membrane blebbing, cytoplasmic shrinkage and reduction of cellular volume, condensation of the chromatin, and fragmentation of the nucleus, all of which ultimately lead the formation of apoptotic bodies, a prominent morphological feature of apoptotic cell death (Kroemer et al., 2005). The caspases, a family of cysteine proteases, play a central role in most apoptotic processes constructing the protease cascade including the initiator caspases (caspase-8 and -9) and the effector caspases (caspase-3, -6 and -7) (Taylor et al., 2008). It has been also highlighted the correlation between the chemical potential for the induction of apoptosis and carcinogenesis (Holme et al., 2007). The fate of cells with DNA damage either to undergo apoptosis or to survive seems to be dependent on the intensity of DNA damage. When weak DNA damage was induced, the cellular response allows repair of the damage. However, if the damage failed to be repaired, mutagenic lesions could be propagated and might lead to carcinogenesis.

Numerous studies have demonstrated that food mutagens (Hashimoto et al., 2001, 2004; Salas and Burchiel, 1998; Shiotani and Ashida, 2004) and tobacco specific

* Corresponding author. Tel.: +34 91 394 37 47; fax: +34 91 394 37 43.
E-mail address: hanais@vet.ucm.es (A.I. Haza).

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Nomenclature

(H_2DCFDA) 2′, 7′-dichlorodihydroflourescein diacetate
(MTT assay) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
(AO) Acridine orange
(FC) Flow cytometry
(NAC) N-Acetyl-L-cysteine
(NOC) N-Nitroso compounds
(NDBA) N-Nitrosodibutylamine
(NDMA) N-Nitrosodimethylamine
(NPIP) N-Nitrosopiperidine
(NPYR) N-Nitrosopirrolidine
(PARP) Poly (ADP-ribose) polymerase
(ROS) Reactive oxygen species
(TUNEL assay) TdT-dUTP Terminal Nick-End Labeling

N-Nitrosamine (Tithof et al., 2001) induce apoptosis. Our previous work also reported that NPIP and NDBA-induced apoptosis in human leukemia HL-60 cell line (García et al., 2008). However, the liver is its major target for carcinogenesis, since alkylating species is produced in hepatocytes (Mirvish, 1995). Numerous *in vitro* studies have employed human hepatoma HepG2 cells to characterize the apoptotic programmed cell death (Kim et al., 2006; Matsuda et al., 2002), becoming a very useful tool for the study of the apoptotic effect of several hepatocarcinogens (Chen et al., 2003; Panaretakis et al., 2001). Thus, the aim was to investigate the induction of apoptosis by NPIP and NDBA in the human hepatoma cell line (HepG2).

As well as DNA damage constitutes the primary signal for the induction of apoptosis, others mechanisms such as oxidative stress may play an important role during apoptosis induction (Chandra et al., 2000). N-Nitrosamines may cause the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury (Bansal et al., 2005; Yeh et al., 2006). For that reason, we also asked whether the induction of apoptosis in HepG2 cells by NPIP and NDBA is mediated by a ROS-dependent cell death pathway.

2. Material and methods

2.1. Chemicals

N-Nitrosopiperidine (NPIP), N-Nitrosodibutylamine (NDBA), Dimethyl sulfoxide (DMSO), Etoposide, N-Acetyl-L-cysteine (NAC) and Acridine orange (AO) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Culture medium and supplements were purchased from Gibco Laboratories (Life Technologies, Inc., Gaithersburg, MD 20884-9980). 2′, 7′-dichlorodihydroflourescein diacetate (H_2DCFDA) was obtained from Molecular Probes (Eugene, Oregon, USA). The caspase inhibitors, Z-DEVD-FMK (caspase-3 inhibitor), Z-VEID-FMK (caspase-6 inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor) were purchased from BD Pharmigen (USA) and dissolved at 10 mM in DMSO (0.1%). All other chemicals and solvents were of the highest grade commercially available.

2.2. HepG2 cells

Human hepatoma cells (HepG2) were obtained from the Biology Investigation Center Collection (BIC, Madrid) and maintained in Dulbeccós Modified Eaglés Medium supplemented with 10% v/v heat-inactivated foetal calf serum, 50 μg/ml streptomycin, 50 UI/ml penicillin and 1% v/v L-Glutamine at 37 °C humidified atmosphere containing with 5% CO_2. Controls included a medium control without N-Nitrosamines as negative control. Etoposide has been extensively studied (Custódio et al., 2002) and was used here as a positive control (100 μM) of apoptosis.

2.3. Morphological evaluation of cell death

HepG2 cells (1×10^6/ml) were treated with NPIP (10–45 mM) or NDBA (1–3.5 mM) at different incubation times. After treatments, cells were stained with acridine orange (5 μg/ml) for 10 min and observed by fluorescence microscopy (Axiostar plus microscope, Zeiss) as described by Gregory et al. (1991). A total of 200 cells were counted in multiple randomly selected fields, and the percentage of apoptotic cells was calculated.

2.4. TdT-dUTP Terminal Nick-End Labeling (TUNEL) assay

Apoptotic cell death was also measured by the In Situ Cell Death Detection Kit, Fluorescein according to the manufacturer's protocol (Roche, Indianapolis, USA). HepG2 cells were treated with NPIP (10, 25 and 45 mM) or NDBA (1, 2.5 and 3.5 mM) for 24, 48 and 72 h. When NAC was used, cells were pre-incubated with 20 mM NAC for 1 h and exposed to N-Nitrosamines. Briefly, 3×10^6 cells were washed with PBS and fixed in 2% formaldehyde in PBS (1 ml) for 1 h at room temperature. The cells were permeabilized with 0.1% triton