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Exposure to dibenzofuran triggers autophagy in lung cells

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ABSTRACT

Environmental pollutants, such as dioxins and furans, are extremely toxic and related with pulmonary disease development. Exposure of A549 human lung cells to dibenzofuran showed both time- and concentration-dependent decreases in cell proliferation and MTT reduction, but no alterations in cell viability. No differences were observed in the number of apoptotic nuclei, which can be due to the energetic failure caused by dibenzofuran-induced ATP depletion. Moreover, cells in culture exposed to the pollutant showed an increase in the conversion of LC3, a protein involved in the autophagic process. Incubation of A549 lung cells with dibenzofuran caused an increase in Lysotracker Red staining, indicating an increase in lysosomal vacuoles content. These results suggest that exposure to dibenzofuran affects lung mitochondrial phosphorylative function, causing an increase in the population of dysfunctional mitochondria and an impairment in the energetic status maintenance, therefore stimulating autophagy as a possible rescue mechanism in this cell line.

1. Introduction

Dioxins and other environmental pollutants are toxic to useful species and to man himself. In humans, exposure to these pollutants, such as fuel constituents, is associated with obstructive pulmonary disease development and with lung cancer. Moreover, chronic respiratory diseases are one of the major problems concerning public health subjects. Dibenzofuran (DBZ) is an aromatic ether with properties and chemical structure similar to dioxins. Potential exposure to dibenzofuran may occur through inhalation and dermal contact, particularly at sites engaged in combustion/carbonization processes (coal tar and coal gasification) where dibenzofuran is released to the ambient air. This pollutant has also been identified in tobacco smoke and listed as a pollutant of concern due to its persistence in the environment, potential to bioaccumulation and toxicity to humans and the environment. Nevertheless, and although the lung is a primary site of exposure for many inhaled chemical contaminants, studies about pollutants-induced toxic effects in lung function are scarce.

Mitochondrial dynamics is clearly important in cellular homeostasis. Mitochondria are the energy suppliers for eukaryotic cells and a proper mitochondrial function is therefore essential in the fulfilment of the tissues energy-demand. ATP synthesis occurs via electron transport and oxidative phosphorylation; for that reason, mitochondria are also involved in the production of reactive oxygen species (ROS) (Carlson et al., 2005). Therefore, alterations of mitochondrial bioenergetic features by toxicants reduce energetic charge and result in cell death. Previous studies have identified mitochondrial targets of environmental pollutants, namely ROS production and decreased ATP content (Senft et al., 2002; Shertzer et al., 2006). Consequently, maintenance of cell function is strictly dependent on the existence of a healthy population of mitochondria. In this respect, mitochondrial degradation by autophagy (mitophagy) may play an essential role in maintaining mitochondrial functionality.

Autophagy is an evolutionarily conserved subcellular degradation process decomposing folded proteins, protein complexes and entire organelles, such as aggregates of misfolded proteins or damaged mitochondria (Gozuacik and Kimchi, 2007). Classically considered to be a pathway contributing to cellular homeostasis and adaptation to stress (e.g. during times of starvation), the autophagic machinery is now recognized to be recruited during several pathogenic conditions and diseases, and during the execution of a caspase-independent cell death (Levine and Kroemer, 2008; Rubinsztein et al., 2007).

The main objectives of this work concerned dibenzofuran effects on pulmonary cellular function. A human epithelial lung cell line exposed in culture to dibenzofuran was used to address cell death and viability, ATP content, mitochondrial membrane potential and ROS production, nuclear morphology and autophagy induction.

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Clarifying the role of pollutants in some mechanisms of toxicity, such as unbalance of bioenergetics status or cell death induction, may help to explain the progressive and chronic evolution of lung diseases.

2. Materials and methods

2.1. Materials

Dibenzofuran was purchased from Supelco, USA (4-0261). Sulforhodamine B (TOX-6), MTT (TOX-1), 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA) (D6883), and lactate dehydrogenase (LDH)-based kit (TOX-7) were purchased from Sigma. Tetramethylrhodamine methyl ester (TMRM) (T668), LysoTracker Red DND-99 (L7528) and Hoecst 33342 (H1399) were purchased from Invitrogen.

All other reagents and chemicals used were of the highest grade of purity commercially available.

2.2. Antibodies

Mouse monoclonal antibody to LC3 (microtubule-associated protein 1 light chain 3B) was purchased from NanoTools, Germany (0260-100/LC3-2G6); mouse anti-β-actin antibody from Sigma, USA (A5441); guinea-pig anti-p62 antibody from Progen, Germany (GP62-C); AP-conjugated goat anti-mouse antibody from Amersham Biosciences, UK (NIF 1316) and HRP-conjugated rabbit anti-guinea-pig antibody from Zymed (61-4620).

2.3. Cell line

A549 human lung carcinoma cell line was purchased from American Type Culture Collection, USA (ATCC catalog no. CCL-185).

2.4. Cell culture

A549 cells were cultured in Minimum Essential Medium (MEM) Eagle (with 1.5 g/l sodium bicarbonate and 0.11 g/l pyruvate), containing 10% fetal bovine serum (FBS). Cells were maintained in a humidified CO_2 (5%) incubator at 37 °C, and passaged and harvested for experiments by detachment with a recombinant enzyme trypsin, TrypLE Express (Invitrogen, Cat. No. 12605). 80–85% confluent cells were used for DBZ treatment. Control cells received an equivalent amount of vehicle (ethanol 0.1%).

2.5. Cell death/viability

For analysis of cell death 2.5×10^4 A549 cells were grown in 12-well plates and treated as indicated 24 h after seeding. When indicated, cells were collected and stained with propidium iodide and annexin-V-FLUOS (BenderMedSystems) according to the manufacturer's protocol. Cell viability was then measured by flow cytometry (FACSCalibur, BD Biosciences) as the percentage of annexin-V- and propidium iodide-negative events.

2.6. Cell proliferation

Cell proliferation and in vitro cytotoxicity were measured with Sulforhodamine B method (Skehan et al., 1990). The cells were seeded in 12-well plates at 1.25×10^4 cells/ml (in a total medium volume of 2 ml per well) and allowed to attach and recover for 1 day prior to drug treatment. After addition of DBZ cells were cultured for up to 2 days (24 h and 48 h). After the treatment, cells were briefly washed, fixed and stained with the dye. The incorporated dye was then liberated from the cells in a Tris-base solution. Absorbance of the solubilized dye was measured at 540 nm in a Victor^3 plate reader (Perkin–Elmer), indicating the degree of cytotoxicity caused by test compound.

2.7. LDH leakage

Cell viability and membrane integrity were evaluated by measuring the amount of cytoplasmic LDH released into the medium, using an assay based on the reduction of NAD by the action of LDH; the resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically at 490 nm. A549 cells were seeded in 12-well plates at 1.25×10^4 cells/ml and allowed to attach and recover for 1 day prior to drug treatment. After addition of DBZ cells were cultured for up to 2 days (24 h and 48 h). Medium from each well (each experimental sample) was centrifuged to pellet cells and aliquots were transferred to clean flat-bottom plate to proceed with enzymatic analysis accordingly to the in vitro toxicology assay kit.

2.8. MTT reduction assay

Mitochondrial dehydrogenases activity was evaluated by measuring the amount of formazan formed after cleavage of the MTT tetrazolium ring. A549 cells were seeded in 12-well plates at 1.25×10^4 cells/ml (final volume of 2 ml) and allowed to attach and recover for 1 day prior to drug treatment. After addition of DBZ, cells were cultured for up to 2 days (24 h and 48 h). At the end of the treatment, 1 mg of MTT (from a recently prepared stock of 5 mg/ml in PBS) was added to each well and the plates returned to the incubator for 3 h to allow MTT reduction. After the incubation media was dumped off and purple formazan crystals were dissolved in 1 ml isopropanol during 30 min on a shaking table. The resulting purple solution was spectrophotometrically measured at 540 nm.

2.9. Adenine nucleotides content

A549 cells were cultured in flasks and treated for 24 and 48 h with DBZ 150 μM and 250 μM. At the end of the treatment cells were harvested by detachment with trypsin; adenine nucleotides were extracted using an acid extraction ($HClO_4$) procedure, final pH 7 achieved with aliquots from stock 2.5 M KOH in 1.5 M K_2HPO_4, maintaining samples on ice. Adenine nucleotides were separated by reverse-phase high-performance liquid chromatography, HPLC (Stocchi et al., 1985). The chromatographic apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector, controlled by a computer. The detection wavelength was 254 nm, and the column was a 5-m Lichrospher 100RP-18 from Merck (Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer (KH_2PO_4, pH 6.5) and 1.2% methanol was performed with a flow rate of 1 ml/min. The time required for each analysis was 5 min.

2.10. Measurement of mitochondrial membrane potential ($\Delta\Psi$)

Mitochondrial membrane potential in A549 cells was measured with a fluorescent probe, tetramethylrhodamine methyl ester (TMRM), as described before (Rolo et al., 2003) with slight modifications. Briefly, to monitor mitochondrial $\Delta\Psi$, cells grown in 12-well plates were loaded with 6.6 μM TMRM for 15 min at 37 °C. Cells were then washed and culture medium without FBS or phenol red was added. TMRM is a cell-permeant, cationic fluorescent dye that is readily sequestered by active mitochondria, accumulating electrophoretically in proportion to their $\Delta\Psi$ (Ehrenberg et al., 1988). Fluorescence was measured using excitation and emission wavelengths of 485 and 590 nm, respectively. After recording basal fluorescence, mitochondrial $\Delta\Psi$ was estimated taking into account the complete depolarization caused by 2,4-dinitrophenol (DNP), normalizing the data to protein content in each well.

2.11. Measurement of ROS production

ROS production was determined fluorometrically, as described before (Palmeira et al., 2007). Briefly, cells were collected by trypsinization and centrifugation, and resuspended at 1×10^6 cells/ml in culture medium without FBS or phenol red. Cells were loaded with 50 μM H_2DCF-DA for 30 min at 37 °C, washed and 200 μl containing 2×10^5 cells were loaded into a 96-well plate. The fluorescence coming from the formation of oxidized derivatives was monitored at an excitation wavelength 485 nm and an emission wavelength 538 nm, for 30 min, to calculate the rate of ROS formation.

2.12. Nuclear morphology (apoptosis)

Cells were seeded on glass cover slips in 6-well plates at 1.25×10^4 cells/ml, as previously described (in a total medium volume of 5 ml per well). At 24 h or 48 h treatment (250 μM dibenzofuran), plates were rinsed twice with PBS. Cover slips were fixed in solution (1 ml) of paraformaldehyde 4% and sucrose 4% during 15 min at room temperature. Cells were then washed two times with PBS. The cells were stained with 5 μg/ml Hoechst 33342 in PBS during 10 min to evaluate nuclear morphology. Cover slips were mounted in a fluorescent mounting medium (Dako) and images were recorded using a Zeiss Axioskop 2 Plus fluorescence microscope (Carl Zeiss) with a Zeiss AxioCam MRC (Carl Zeiss). Apoptotic cells were identified as those whose nuclei exhibited brightly staining condensed chromatin, nuclear fragmentation or apoptotic bodies.

2.13. Lysotracker accumulation

Cells were seeded on glass cover slips in 6-well plates as described above. After DBZ treatment they were gently washed with PBS and incubated for 30 min with LysoTracker Red 100 nM in culture medium without phenol red. Cells were then inspected and photographed using a Zeiss Axioskop 2 Plus fluorescence microscope (Carl Zeiss) with a Zeiss AxioCam MRC (Carl Zeiss).

2.14. LC3 subcellular distribution

A549 cells were seeded on glass cover slips in 6-well plates as described above, and transfected with YFP-LC3 plasmid (kindly provided by Dr. M. Sandri in the Venetian Institute of Molecular Medicine, Padua, Italy) using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions. Cells were allowed to express the protein and incubated with dibenzofuran 24 h after transfection. As a positive control for autophagy induction cells were incubated in "starvation" medium (Hank's Balanced Salt Solution supplemented with 10 mM