Original Article

Halothane Does Not Directly Interact with Genome DNA of A549 Cells

(A549 / anaesthetics / DNA damage / halothane / lung cells)

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Abstract. Although the inhalation anaesthetics are commonly used in clinical practice, their toxic effects on the lung cells have not yet been well studied. Previous studies indicated strong genotoxic effect of some inhalation anaesthetics, applied at clinically relevant concentrations. The aim of the present study was to assess the extent of DNA damage, nuclear abnormalities and possibility of human A549 cells to recover after treatment with halothane at lower concentrations. The data obtained demonstrate that even lower halothane concentrations could induce DNA damage although the anaesthetic does not interact directly with DNA. We have found that irreversible impairment of the cell genome is initiated at a concentration as low as 1.5 mM. Part of the cell population displays some characteristics of stress-induced apoptosis, defining this concentration as threshold for cell survival. We suggest that the intracellular signalling pathway triggers the toxic effects of halothane.

Introduction

Inhalation anaesthetics are commonly applied in surgery, particularly in paediatric practice (Walpole et al., 2001) and in adult patients for their vasodilatation effect (Stimpfel and Gershey, 1991; Su and Vo, 2002). Postoperatively, both age groups are at risk to develop functional disturbances of pulmonary surfactant system implicated in respiratory distress syndrome (Haitsma et al., 2003; Lalchev, 2006). Pneumocytes type II are the main producers of surfactant components. They also play a role in tissue turnover and recovery after lung injury as alveolar progenitor cells (Lalchev, 1997). Elucidation of the mechanisms of lung cell damage after inhalation could be of particular significance for the medical practice.

Halothane is one of the widespread volatile anaesthetics, introduced in medical practice in 1956 to substitute chloroform and ether. It exerts faster recuperative effect after anaesthesia and less inflammatory incidences of the lung tissue (Habre et al., 2001). Similarly to other halogenated ethers, halothane affects the ion balance, impairs membrane-bound proteins, cell functions, and could irreversibly damage many intracellular structures (Molliex et al., 1998; Juvin et al., 1999; Kaech et al., 1999). Reduction in phosphatidylcholine production and total phospholipid content are found after exposure of cultured cells to halothane (Molliex et al., 1994; Valtcheva et al., 2003). Ultrastructural alterations of the cells studied and a decline in their adhesive properties are observed when concentrations of the anaesthetic above 2.1 mM are used (Valtcheva et al., 2003, Topouzova-Hristova et al., 2006). In animal organ model systems, reduction of surfactant secretion by pneumocytes type II (Kaech et al., 1999; Allaouchiche et al., 2001; Lalchev et al., 1992) and low cytosolic levels of ATP (Patel et al., 2002) have been reported. Recent data have pointed out that exposure of the lung to sevoflurane and desflurane leads to oxidative stress (Allaouchiche et al., 2001). Our previous studies on two lung-derived cell lines (A549 alveolar cells and 14HBE16o$^-$ bronchial cells) showed a pronounced cytostatic and cytotoxic effect of penthrane (0.5% and 1% v/v) and halothane (1% and 2% v/v) (Topouzova et al., 2003a). The genotoxic effect of penthrane on both cell lines was evidenced by alkaline comet assay indicating higher sensibility of A549 cells than of the bronchial cells (Topouzova et al., 2003b). It should be emphasized, however, that pen-

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Abbreviations: DMEM – Dulbeco's modified Eagle's medium; DSB – double-strand breaks; FBS – foetal bovine serum; MAC – minimal alveolar concentration; NORs – nucleolar organization regions; PBS – phosphate-buffered saline; TBA – Tris-borate-acetate; TFA – trifluoroacetic acid; UV-VIS – ultraviolet-visible.

thrane treatment led to more severe impact on the cells studied than halothane. Clinical practice has recorded higher frequency of lung post-operative inflammation and nephrotoxicity after penthrane anaesthesia. The cases of nephrotoxicity could be explained with a specific metabolic pathway suggested by Kharasch et al. (2006). Other experiments also provide evidence for the genotoxic and mutagenic effects of inhalation anaesthetics. DNA damage induced by halothane, isoflurane and other inhaled gases has been registered in isolated human peripheral lymphocytes either *in vitro* or as a result of occupational exposure (Sardas et al., 1998; Jałoszynski et al., 1999; Szyfer et al., 2004). Despite all these findings, the molecular mechanisms of halothane effects on lung cells are still unknown.

In the present study, DNA damage and nuclear abnormalities were assessed after applying lower halothane concentrations than those reported previously to have a strong genotoxic effect. The restoration capabilities of the alveolar A549 cells after treatment were also studied. The possibility of halothane to interact directly with DNA was also investigated in an attempt to shed some light on the mechanisms underlying halothane toxicity. Our results demonstrate the absence of direct interaction with DNA and indicate that the irreversible impairment of the cell genome is initiated at a concentration as low as 1.5 mM, which we define as threshold for cell survival. We suggest that an intracellular signalling pathway triggers the toxic effects of halothane.

Material and Methods

Cell culture

All experiments were performed with A549 (from National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria), a lung-derived human carcinoma cell line that maintains many of the morphological and biochemical characteristics of pneumocytes type II (Lieber et al., 1976). The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (FBS) (HyClone, Thermo Fisher Scientific South Logan, UT) and Antibiotic-Antimycotic solution (Gibco BRL, Invitrogen Corporation, Carlsbad, CA), at 37°C, 5% CO_2 until they reached 80% confluence.

The cells were treated with halothane (Narcotane, Leciva, Praha, Czech Republic) concentrations of 0.9, 1.2 and 1.5 mM in DMEM. The anaesthetic was dissolved in the culture medium just before treatment and cells were kept in a closed system (Corning flasks or Parafilm M-sealed dishes) for 2 h at 37°C. The concentration of halothane in the culture medium was determined by UV-VIS spectra as described by Valtcheva et al. (2003). After treatment the cells were washed with phosphate-buffered saline (PBS), pH 7.4, and were maintained for up to five days in a medium without anaesthetic. Untreated cells were used as a control.

Analysis of genome DNA integrity

To assess DNA damage in the whole cell population, electrophoretic analysis of genome DNA was applied. Immediately after treatment with the above-mentioned concentrations the cells were lysed and genome DNA was purified with NucleoSpin kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). To evaluate the repair capability after treatment the cells were washed with PBS, divided into five equal aliquots and allowed to grow without anaesthetic. The genome DNA was isolated every 24 h and DNA injury was estimated by agarose gel electrophoresis (1% agarose, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in Tris-borate-acetate (TBA) buffer (Sambrook et al., 1989). To assess direct interaction of halothane with DNA, purified genome DNA was incubated with either pure anaesthetic for 120 min, or solution of the anaesthetic in culture medium for 10 min. The applied concentrations were adjusted mixing DNA aliquots with the appropriate volume of 3 mM halothane stock solution in DMEM. DNA integrity was examined as described above.

Nuclear and nucleolar staining

Cells grown on coverslips were treated with halothane at concentrations mentioned above for 2 h and allowed to recover for several days in fresh medium. Samples were taken every 24 h, washed twice with PBS, fixed with Carnoy fixative for 30 min and stained *in situ* for 5 min with May-Grunwald-Giemsa solution (BioOptica, Milano, Italy) or Ag-NOR kit (BioOptica, Milano, Italy) The slides were analysed with Olympus CX21 microscope (Olympus Deutschland GmbH, Mainz, Germany). Cells with nuclear abnormalities, such as fragmented nuclei, bi- and trinucleated cells were counted and expressed as percentage of the total cell population. Each experiment was performed in triplicate and data were presented as a mean ± SE.

Statistical analysis

Each experiment was performed in triplicate and data were presented as a mean ± SEM. Genome integrity experiments were repeated seven times. Statistical analysis was performed by one-way ANOVA using the GraphPad InStat 3.01 software (GraphPad Software, Inc., San Diego, CA). Tukey's and Dunnett's tests were used to statistically estimate the differences between individual experimental groups. The data also passed the Kolmogorov-Smirnov normality test.

Results

The electrophoretic analysis of genome DNA, isolated immediately after 2 h of treatment with concentrations ranging from 0.9 to 1.5 mM halothane, revealed a dose-dependent genotoxic effect on A549 cells. A significant positive correlation between the accumulation of DNA lesions and the applied doses was observed

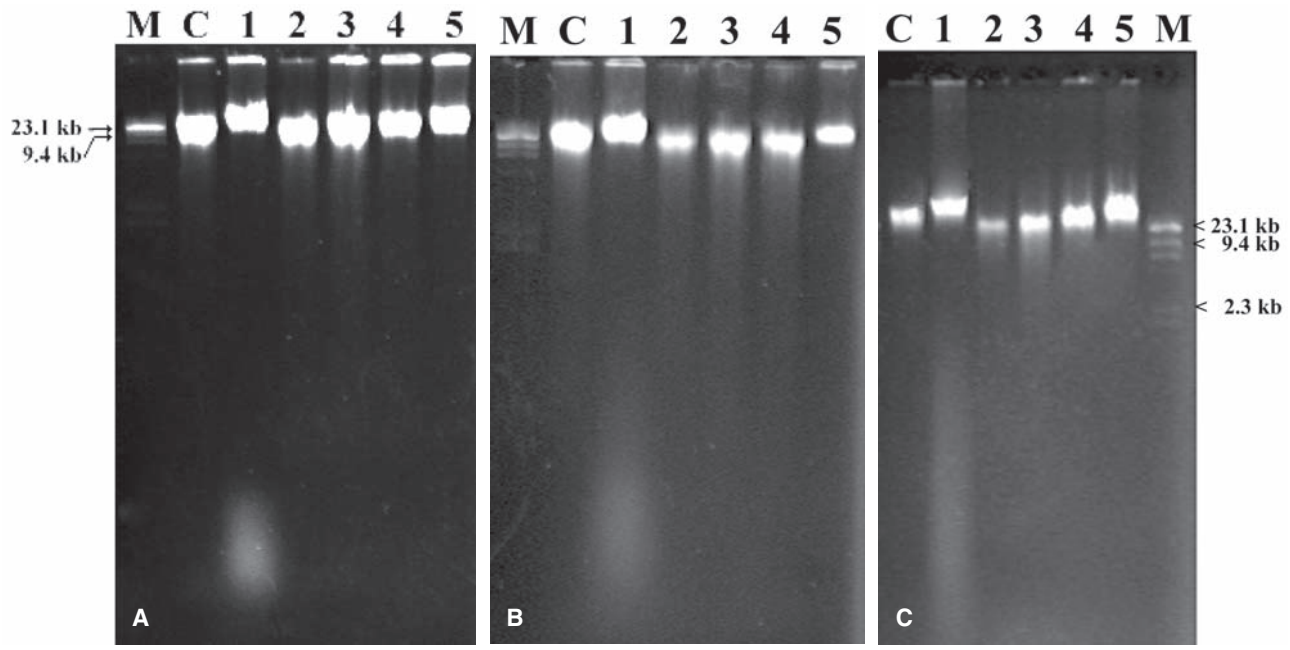

Fig. 1. Partial repair of damaged DNA after 2-h treatments with halothane. The genome DNA from untreated cells (control, C) is compared with DNA isolated immediately (line 1) and from the first to the fourth day after treatment (lines 2-5) with 0.9 mM (panel **a**), 1.2 mM (panel **b**) and 1.5 mM (panel **c**) halothane. M – molecular weight marker (λ / *Hind*III).

(Fig. 1). The immediate effect of the anaesthetic was manifested as dose-dependent alterations on cell periphery, i.e. 10–30 % of the cells were detached during the treatment, which corresponded to the decreased amount of DNA (Fig. 1c, line 2). These results confirmed previously published data from LDH test as well as the cell detachment/attachment alterations after exposure to halothane (Valtcheva et al., 2003).

The repair capability of the cells was verified by examination of the dynamics of DNA injury and recovery in cells exposed to halothane concentrations of 0.9, 1.2 and 1.5 mM (Fig. 1a-c). The genome DNA was isolated every 24 h and analysed by neutral electrophoresis. Slight smears immediately after treatment and gel shift of the residual DNA amount above the control DNA cells were detected in all samples (line 1 in Fig. 1a-c). These results suggested that halothane induced accumulation of double-strand breaks and formation of high-molecular DNA fragments probably by intermolecular crosslinks. The amount of low-molecular fragments gradually declined in the following days and finally disappeared, while high-molecular DNA fractions moved around 23 kb on the first day after treatment (line 2 in Fig. 1a-c). In all samples, DNA repair was observed mainly on the third and fourth days after the treatment (Fig. 1a-c, lines 4 and 5).

Other experiments were carried out to follow the influence of DNA lesions on the nuclear and nucleolar morphology. As denoted in Fig. 2, halothane affected nuclear structures in a dose-dependent manner (Fig. 2a). Significant differences were observed up to a concentration of 1.5 mM ($P < 0.01$). At this critical point, nuclear abnormalities increased threefold up to the end of the experimental period (Fig. 2a). The lower concentrations induced similar changes on the first 1–2 days, and in the later period the percentage of affected cells remained lower compared with the samples exposed to 1.5 mM. Pycnotic and fragmented nuclei, abnormal mitoses and multinuclear cells were identified (Fig. 2b). Surprisingly, the concentration of 1.5 mM also produced an increase in the number of nucleolar organization regions (NORs) (detected by AgNOR staining) to the values above those in untreated cells (Fig. 2c). However, it was not possible to detect differences in the number of NORs at lower concentrations (not shown).

To verify whether halothane interacts directly with DNA, purified genome DNA was incubated for 10 min with concentrations ranging from 0.15 to 1.5 mM halothane. The electrophoretic analysis showed that all samples contained high-molecular genome DNA without indications of fragmentation (Fig. 3a). In addition, two-hour incubation with 100% halothane did not result in DNA degradation (Fig 3b). These results suggested that the above-mentioned DNA lesions could be due to either the metabolic activation or the activation of an intracellular signalling pathway. The latter option is more likely since primarily the liver metabolizes 15% of the inhaled halothane, while the major part is exhaled unchanged through the lung (Holaday, 1977). Thus, metabolic activation could be responsible for hepatotoxicity, but not for lung cytotoxicity.

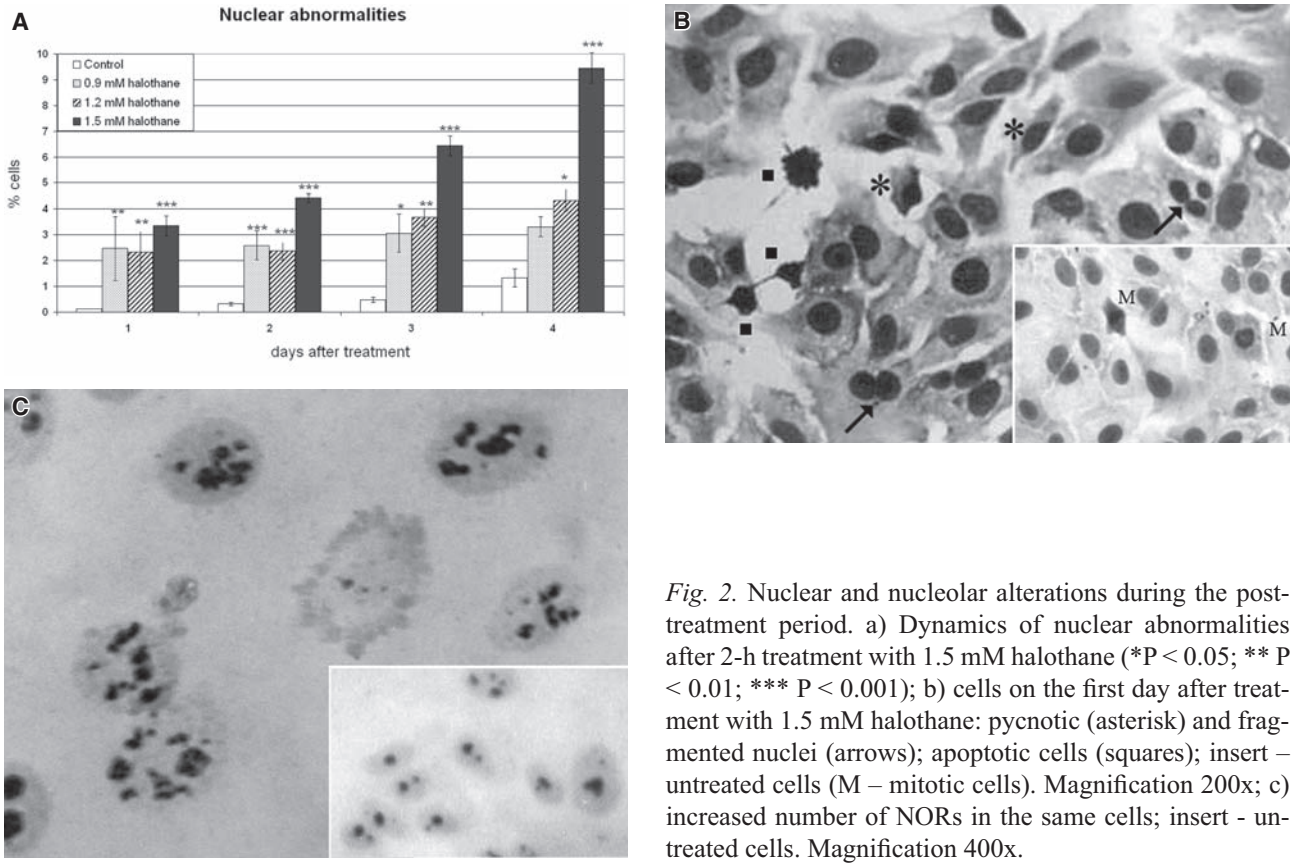

Fig. 2. Nuclear and nucleolar alterations during the post-treatment period. a) Dynamics of nuclear abnormalities after 2-h treatment with 1.5 mM halothane (*P < 0.05; ** P < 0.01; *** P < 0.001); b) cells on the first day after treatment with 1.5 mM halothane: pycnotic (asterisk) and fragmented nuclei (arrows); apoptotic cells (squares); insert – untreated cells (M – mitotic cells). Magnification 200x; c) increased number of NORs in the same cells; insert - untreated cells. Magnification 400x.

Discussion

Potential toxicity of the present inhalation anaesthetics (halothane, isoflurane, sevoflurane, desflurane and enflurane) is usually associated with their metabolic products (such as trifluoroacetic acid (TFA), Br-, Cl-conjugates of TFA, compound A, inorganic F, etc.) although their molecules are highly fluorinated, and thereby with increased molecular stability (Kharasch et al., 2006). The major metabolite of halothane – trifluoroacetic acid – is relatively non-reactive and is believed to be

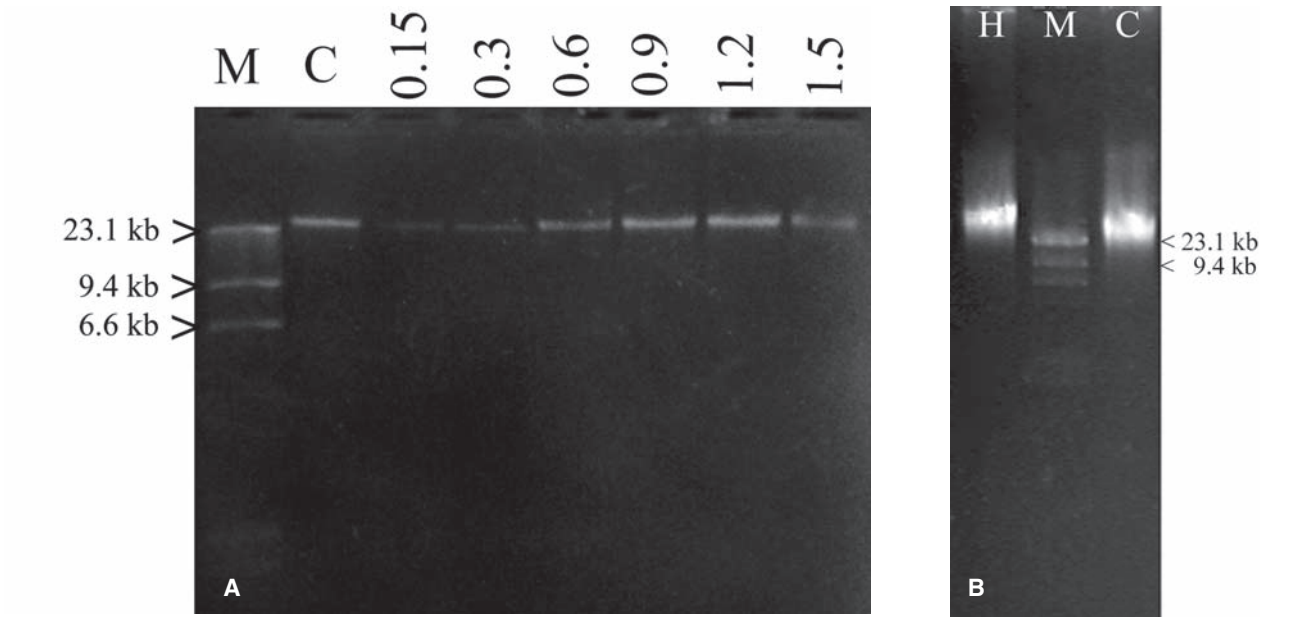

Fig. 3. Isolated genome DNA treated with halothane. a) Purified DNA treated with halothane at concentrations indicated above for 10 min; C – untreated control; M – molecular weight marker (λ / *Hind*III); b) purified DNA treated with 100% halothane for 2 h (H); molecular weight marker (M); untreated control (C).

non-toxic. However, the oxidative or reductive defluorination pathways could lead to the formation of cytotoxic and genotoxic metabolites (Kharasch et al., 2006). Important contributions in clarifying the impact of anaesthetics are data indicating their cyto- and genotoxic effect in a different model system (Molliex et al., 1998; Sardas et al., 1998; Jałoszynski et al., 1999; Patel et al., 2002; Topouzova et al., 2003 a, b; Valtcheva et al., 2003; Szyfer et al., 2004; Topouzova-Hristova et al., 2006).

Information on genome integrity of lung cells after application of inhaled agents is insufficient at the present time. A549 cells were chosen for our experiments because they are an established model system for *in vitro* studies of pneumocyte type II functions. This cell line keeps the main properties of pneumocytes type II, e.g. alveolar surfactant production (Lieber et al., 1976). Despite their cancer origin, A549 cells possess inherent p53 gene (Fujita et al., 2002) and are also used in studies of apoptotic induction or cytotoxic effect (Lestari et al., 2005; Gualtieri et al., 2005).

Halothane remains an important anaesthetic because of its relatively low cost, although it occasionally induces hepatitis in susceptible patients. In surgery the anaesthetic potency of inhaled agents is expressed in terms of their minimal alveolar concentration – MAC (O'Keeffe and Healy, 1999). MAC of halothane depends on the patient's age and is usually in the range of 0.64–1.8%. In clinical practice halothane is applied at concentrations up to 4%, giving theoretical blood concentration > 4.3 mM (Jałoszynski et al., 1999). During anaesthesia alveoli are subjected to inhalational anaesthetics for time longer than any other cells. Our previous results have shown that 3 mM halothane has severe effect on the lung cells and the bulk of the cell population die (Topouzova-Hristova et al., 2006). Thus, in the present study we report the effect of halothane at lower concentrations between 0.9 and 1.5 mM on alveolar epithelial cells to specify the threshold concentration of the exerted negative effect and to provide additional data on its genotoxicity. The results allowed us to assume that halothane caused dose-dependent DNA damage in alveolar cells and might provoke stress-induced apoptosis even at a concentration of 1.5 mM. The typical apoptotic DNA ladder was not observed by gel electrophoresis (Fig. 1). DNA fragmentation during apoptosis is known to proceed through several consequent stages. Initially DNA is cleaved to 30–50 kb fragments, which do not form a ladder in morphologically normal cells. The apoptotic DNA ladder appears later as a consequence of DNA fragmentation to low-molecular-weight fragments, and is associated with late apoptotic events, such as cell shrinkage and formation of apoptotic bodies (Czene et al., 2002). In our experimental material, after 1.5 mM halothane treatment only 10% of the cells showed nuclear abnormalities which could be evaluated as apoptotic morphological features (Fig. 2a). We consider this concentration to be a threshold since the application of higher concentrations resulted in irreversible changes in cell-adhesive properties (Valtcheva et al., 2003), genomic DNA integrity and apoptosis-like ultrastructural changes in A549 cells (Topouzova-Hristova et al., 2006). This hypothesis is supported by our previous data, indicating that the use of 3 mM halothane leads to the appearance of mini-comets and increased number of severely impaired cells, most likely through the activation of intracellular endonucleases and DNA fragmentation (Topouzova-Hristova et al., 2006). In that report data of DNA damage were obtained by the alkaline comet assay. In principle, the alkaline comet assay reveals all types of genotoxicity, while neutral electrophoresis preferably detects the double-strand breaks (DSB) (Vodicka et al., 2004). In the present study the formation of DSBs and their elimination were followed. Data with lower halothane concentrations demonstrate partial DNA repair, not before the third or fourth day after treatment. A similar genotoxic effect was reported by Jałoszynski et al. (1999) and Szyfer et al. (2004) after the treatment of human peripheral lymphocytes with inhalation anaesthetics. These authors found that cells allowed to recover for 120 min after exposure to 1 mM halothane were able to eliminate the DNA damage incompletely (Szyfer et al., 2004). However, these experiments were carried out at 4°C, when the cellular metabolism is suppressed (Szyfer et al., 2004). In this respect the lymphocytes may be considered as cells that easily overcome the genotoxic effect of halothane. Since A549 cells produce alveolar surfactant, they possess a well-developed intracellular membrane network, which allows faster and easier penetration of the highly lipophilic anaesthetic (Smith et al., 1981; Jałoszynski et al., 1999). Therefore, most probably the halothane affects the intracellular membrane system and hence the cell morphology, which accounts for nuclear and nucleolar abnormalities during the post-treatment period.

The mechanism of induction of DNA damage by halothane remains unclear. One of the possible explanations is direct interaction of halothane with DNA, generating alkaline-labile modifications of purines. Another possibility is the formation of halothane genotoxic metabolites such as TFA, Br- and Cl- conjugates of TFA. Generation of such metabolites via oxidative defluorination by CYP 2E1 is shown in hepatocytes and explains the cases of the so-called halothane hepatitis (Eliasson and Kenna, 1996). On the other hand, the release of active fluor anion from halogenated compounds in aqueous solutions could provoke DNA disintegration *in vitro* (Monig et al., 1983). To check these possibilities, purified DNA was incubated with halothane solutions at varying concentrations. The present results provide strong evidence for the absence of direct interaction between genome DNA and halothane, and the observed effect is most probably a result of an activated signal

transduction pathway (Mitev and Miteva, 1999). The injured cells may further undergo partial repair if treated with anaesthetic concentrations < 1.5 mM. Above this critical point the alterations become irreversible and exhibit some features of stress-induced apoptosis.

We consider the stress-induced apoptosis after exposure to inhalation anaesthetics as very probable. It has now been recognized that there exist different ways of apoptotic induction: extrinsic (receptor-mediated); intrinsic (mitochondria-mediated); and caspase-independent (Orrenius et al., 2003; Klener Jr. et al., 2006). The first option seems inconsistent since it requires specific stimuli such as Fas or TNF-α ligand (Hristov et al., 2006). In contrast, the other pathways can be activated either by specific, i.e. pro-apoptotic proteins, or non-specific stimuli related to outer and intracellular membrane network damage.

In conclusion, our data suggest that the intracellular signalling pathway triggers the toxic effects of halothane. The exact mechanism of halothane effect on the alveolar cell genome is not well understood and further studies are needed to specify the signalling pathways involved. This area of research could contribute to elucidating the role of stress-induced apoptosis in lung diseases and thus to be of value in clinical practice.

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