Rat alveolar type II cells – suitable model for evaluation of cigarette smoke effects

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Cigarette smoke consists of more than 4700 identified constituents. Part of them are free radical substances inducing oxidative damage, which was postulated as a mechanism responsible for the toxicity of smoking. A large amount of cigarette smoke substances has genotoxic effect. Since alveolar epithelial type II cells possess mitotic activity, we studied the possibility to use these cells for testing the genotoxic effect of cigarette smoke in vitro. Micronucleus test was used for assessing the genotoxicity. In parallel, the oxidative stress was evaluated by estimating glutathione and total antioxidant capacity. The epithelial type II cells freshly isolated from rat lung were cultivated on plastic plates. After 20-h cultivation the medium was changed and the cells were cultured in medium treated with various cigarette smoke concentration (0.5–4%). After 24-h cultivation the frequency of micronuclei, the amount of total glutathione and the total antioxidant capacity were evaluated and compared to the cells cultivated in cigarette smoke free medium. The frequency of micronuclei was increased from 4.56 ± 1.06 in 1000 control cells to 42.60 ± 5.20 in 1000 cells cultured 24 h in medium treated with 4% cigarette extract. The total glutathione and total antioxidant capacity were decreased in the same cells to 44 and 21% of the control value, respectively. The results give evidence for toxic and genotoxic effects of cigarette smoke on alveolar type II cells and show the suitability of alveolar type II cells as a model for in vitro genotoxicity studies.

Key words: type II cells, cigarette smoke, micronucleus assay, genotoxicity, oxidative stress, glutathione.

Introduction

Due to its anatomic location, the alveolar epithelium is often directly exposed to inhaled oxidant gases. The direct effect of oxidant stress on type II cells may play a key role in pulmonary toxicity. As type II cells have mitotic activity, they seem to be a suitable in vitro model for detection of genetic damage.

Cigarette smoke is a complex mixture with over 4700 identified constituents (Rahman & Mac Nee, 1996) containing compounds as polycyclic...
antioxidant capacity. The level of glutathione was compared with the total main component of the antioxidant system. The estimating the amount of total glutathione as the cronucleus test for assessing genotoxicity and effect of treatment was evaluated by using the mi-

Cigarette smoke is clastogenic and may disrupt spindle-fiber formation (Rithidech et al., 1989). Induction of spindle or chromosome damage may be detected by micronucleus assay, a widely used short-term screening test developed by Schmid (1975), screening chromosome breaking ability of mutagens by estimating the frequency of micronuclei (MN). MN are formed spontaneously or in response to genotoxic exposure fromacentric chromosomal fragments or whole chromosomes left outside the daughter nucleus during cell division.

Glutathione, a universal constituent of biological systems, is considered to be the most important physiological antioxidant in lung epithelial cells and lung lining fluid. Experimental observations show an association between decreased cellular glutathione and increased susceptibility of lung cells to be damaged by oxidants (Deneke & Fanburg, 1989).

The aim of this study was to establish the suitability of cultured alveolar epithelial type II cells for studying the toxic and genotoxic potential of cigarette smoke. The cells were exposed to fresh cigarette smoke extract obtained by techniques widely used by other workers as a good-validated methods to study the effects of cigarette smoke on various cell functions (Cosgrove et al., 1985; Nakayama et al., 1985; Holden et al., 1989). The effect of treatment was evaluated by using the micronucleus test for assessing genotoxicity and estimating the amount of total glutathione as the main component of the antioxidant system. The level of glutathione was compared with the total antioxidant capacity.

Material and methods
Animals
Male Albino Wistar rats (Velaz, Prague, Czech Republic) weighing 180–220 g were used in these experiments.

Animals were housed under standard laboratory conditions and were given a conventional laboratory diet (MOK, Velaz, Prague, Czech Republic) and tap water ad libitum.

Reagents and media
Dulbecco’s modified Eagle medium (DMEM) was purchased from Pansystem GmbH Aidenbach (Germany), fetal calf serum (FCS) from SEBAK GmbH Aidenbach (Germany), Bio-Rad protein assay from Bio-Rad Laboratories (USA), total antioxidant status was evaluated with Randox kit (Randox Laboratories Ltd., United Kingdom). All other chemicals were supplied by Sigma.

Cell isolation
Rat alveolar type II cells were isolated according to the method of Richards et al. (1987) and Hoet et al. (1994). Briefly, after intraperitoneal sodium pentobarbital (60 mg kg⁻¹) anaesthesia the lung was perfused via the pulmonary artery with sterile saline and was mechanically ventilated. The lung with trachea was removed and bronchoalveolar lavage was performed. The lung was partially trypsinized, chopped and the cell mixture purified by centrifugation on a discontinuous Percoll gradient (density 1.089 and 1.04, 250 × g, 20 min). The interface was collected and after rewashing the cells were resuspended in the medium and plated in a Petri dish. After 1 hour incubation in an atmosphere of 95% air/5% CO₂ the unattached cells were collected and sedimented.

Cell culturing
The cells were cultured on 96-well plates (10 000 cells/200 μl/well) or 24-well plates (30 000 cells/600 μl/well) in DMEM with glutamine supplemented with 10% FCS at 37°C in the humidified atmosphere of 95% air/5% CO₂. The cells for micronucleus test were cultured in 96-well plates and for biochemical analysis in 24-well plates.

Scheme of experiment
The freshly isolated cells were cultured in cigarette smoke free medium. The medium was withdrawn after 20 hour cultivation and the cells were exposed to cigarette smoke-treated medium. The cultivation in the treated medium continued for another 24 hours. Cigarette smoke treated medium was obtained by bubbling the cigarette smoke from one commercially available cigarette (Dalila, S.I.T., Slovak Republic) through 5 mL of DMEM with glutamine supplemented with 10% FCS. The concentrated cigarettes smoke-treated medium was diluted with the medium containing the mentioned supplements and used immediately after preparation.

Micronucleus test
The micronucleus assay was performed according to Liu et al. (1996) with a modification. At the end of the treatment, the cells cultured in 96 well plates were washed twice with phosphate buffered saline, trypsinized at 37°C, washed and cytopsins were prepared (1400 rpm, 6 min). The slides were fixed with
methanol and stained with Giemsa. Micronuclei (MN) were analysed in Jenaval microscope (Zeiss, Germany) at 1000× magnification using oil immersion. The criteria used to score MN followed those reported by Schmid (1975). The results were expressed as an average number of cells with MN per 1000 alveolar type II cells. The frequency of MN was based on 5000–9000 cells scored per group.

Biochemical analysis
After finishing the experiment, the cells cultured in 24 well plates were washed twice with phosphate buffered saline and dissolved in 0.2% Triton X-100.

Glutathione assay. Estimation of total glutathione (GSH) was based on the rate of decrease of 5,5'-dinitrobis-(2-nitrobenzoic) acid measured spectrophotometrically at 412 nm according to the method of Anderson (1985).

Total antioxidant capacity (TAC) was evaluated with the Randox kit (NX 2332). The principle of the assay is based on the colour reaction of 2,2'-azino-di-3-ethylbenzthiazoline sulphonate with peroxidase and H$_2$O$_2$. Antioxidants in the added sample cause suppression of the colour production to a degree, which is proportional to their concentration. For each data point, the mean GSH concentration and antioxidant status of three to four samples was taken.

The protein content was measured using the method of Bradford (1976).

Results
The effects of cigarette smoke treatment on micronuclei frequency in alveolar type II cells are shown in Table 1. The frequency of micronuclei was enhanced and the differences between all exposed groups and the control were statistically significant ($P<0.01$). The number of micronuclei after 24 h treatment was concentration dependent, raising from 10.67 MN/1000 cells to 42.60 MN/1000 cells at 0.5% and 4% cigarette smoke extract, respectively.

The amount of total glutathione was decreased in the cigarette smoke groups compared to the control group. In parallel, the changes of total antioxidant capacity were also measured. The cell exposure to 0.5% cigarette smoke extract reduced the amount of GSH to 88% and the TAC to 78%. The 4% concentration evoked more pronounced effects, the level of GSH was 44% and TAC 21% of the control ones. The decrease in total glutathione and total antioxidant capacity had the same tendency but the absolute value was different (Fig. 1). The changes were statistically significant at 1% and higher smoke concentration ($P < 0.01$).

Discussion
Cultured alveolar epithelial type II cells, according to our knowledge, have not been used before in genotoxic studies. Our results indicate the suitability of this model for assessment of genotoxic potential of the cigarette smoke in vitro. Cigarette smoke exposure resulted also in total glutathione and total antioxidant capacity decrease in type II cells.

The genotoxic effect of cigarette smoke has been confirmed in both in vitro and in vivo systems by micronucleus assay. The results of in vivo experiments suggest that cigarette smoke is clastogenic and may disrupt spindle fibre formation in different cells (Mohitasampur et al., 1987; Rithidech et al., 1989; Blagoevova et al., 1997). The genotoxic action found far distant from the lungs (e.g. bone marrow, fetal liver) should be taken into consideration when the general consequences for the organism of inhaling cigarette smoke.
smoke are assessed. But the lungs still remain the main target organ of the adverse effect of cigarette smoke. Thus our attention was focused on the lung. In this study, cell culture model was chosen in order to eliminate the influence of known effects of cigarette smoke on, for instance, inflammatory cells, and because it facilitated investigation of the mechanisms. Alveolar epithelial type II cells were chosen because of their importance from toxicological point of view and their mitotic activity in culture (Mason & Crystal, 1998). It is rather difficult to compare the results received in various in vitro systems because of different generation modes and concentrations of the cigarette smoke.

The genotoxic effect of cigarette smoke was observed by many authors in different cells using several in vitro assays, for instance in cultured human cells (Nakayama et al., 1985, 1986) in Chinese hamster lung cells (Laft & Parry, 1991; Channarayappa et al., 1992; Massey et al., 1998) and in BALB/c-3T3 cells (Gu et al., 1992). The results of Channarayappa et al. (1992) with cigarette smoke condensate (CSC) in Chinese hamster lung V79 cells suggested the presence of both clastogenic and aneuploidogenic agents in cigarette smoke. However, the higher frequency of KC+ than KC- micronucleated cells was indicative of a stronger aneuploidogenic effect of CSC. CSC induced statistically significant dose dependent increase of MN in BALB/c-3T3 cells (Gu et al., 1992). The results presented in Table 1 confirmed the suitability of rat alveolar type II cells for evaluation of micronuclei induced by cigarette smoke. Micronucleus formation requires one mitotic division and our results confirmed the ability of type II cells to undergo mitosis also in cell culture. A series of experiments were carried out to assess the mutagenic effect of the cigarette smoke on the type II cells resulting from 24-h exposure. Highly significant smoke-induced differences in MN frequencies were observed in the exposed cells versus controls. Our data provided evidence on the mutagenicity of cigarette smoke and on the suitability of our method for observing the induction of MN caused by cigarette smoke exposure in vitro. It may serve as an appropriate adjunct for the in vitro toxicological assessment of cigarette smoke and other aerosols (Chihavatovičová et al., 2000).

While the mechanisms involved are not yet clear, free radical-induced oxidative damage has been suggested to play a role in the pathogenesis of smoking related disorders. Reactive oxygen species are capable of reacting and inactivating biological materials, including single strand breaks in DNA, inactivating protein/enzymes, and oxidising lipids. Thus, cigarette smoke exposure may cause oxidative damage (Wurzel et al., 1995). Glutathione is an important physiological antioxidant in lung epithelial cells and lung lining fluid and plays crucial roles in protecting proteins and DNA from oxidation caused by cigarette smoke (Park et al., 1998). Enormous amounts of free radicals and reactive oxygen species are produced during cigarette smoking and these compounds are suggested to react with protein thiols and presumably with small thiols such as glutathione (Eiserich et al., 1995). Oxidative damage can result not only from a direct reaction with substances present in cigarette smoke but also from smoke-induced secondary agents. Park et al. (1998) described depletion of the glutathione pool in the lung of rats after inhalation exposure. These results from in vivo experiments are in concordance with our results, where the glutathione depletion was both concentration and time (unpublished data) dependent. The pulmonary tissue is normally protected by a number of enzymatic and nonenzymatic antioxidant systems. The diminishing total antioxidant capacity in type II cells after cigarette smoke treatment in our experiments was parallel to the decreasing amount of GSH, suggesting that the protective effect of GSH cannot be substituted by any other component of the antioxidant system.

In summary, the results of this study demonstrate the suitability of cultured alveolar epithelial type II cell for the assessment of both genotoxic and toxic studies of cigarette smoke.

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References


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