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Concentration of TBA-reactive substances in type II pneumocytes exposed to oxidative stress

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Introduction:

Oxidative lung damage may be associated with the destruction of alveolar cells. Type II alveolar epithelial cells (AECs), as progenitors of type I cells, are indispensable for the renovation of alveolar structure after lung injury. Extensive damage to type II cells could be responsible for unfavorable outcome. However, the susceptibility of type II AECs to oxidative stress is unclear.

Materials and Methods:

We investigated the susceptibility of freshly isolated and cultured rat type II AECs to oxidative stress (H_2O_2 and Fe^{2+}). Thiobarbituric acid reactive substances (TBARS) were measured as indices of lipid peroxidation and cytotoxicity was estimated by the MTT test. Aminotriazol (ATZ), an inhibitor of intracellular catalase, was used to estimate the protective role of catalase.

Results:

TBARS concentration increased significantly in freshly isolated, oxidant-exposed cells (4.0 ± 1.3 vs. 8.3 ± 2.2 nmol/g protein, $p=0.0313$) and insignificantly in cultured cells (1.7 ± 0.4 vs. 4.4 ± 1.7 nmol/g protein). ATZ was toxic even to cells not exposed to oxidants. Inhibition of catalase in cells exposed to oxidants resulted in an insignificant increase in TBARS: 4.5 ± 1.5 vs. 16.2 ± 3.9 nmol/g protein, $p=0.0625$, and 4.0 ± 0.8 vs. 7.6 ± 4.0 for freshly isolated and cultured cells, respectively. Oxidative stress itself did not increase cytotoxicity.

Conclusions:

Type II AECs are not resistant to oxidative stress. We cannot, however, explain why cells with evidence of lipid peroxidation do not show increased cytotoxicity. The toxicity of ATZ is not related to oxidative cell damage. In cells exposed to oxidants, TBARS may further increase when catalase is inhibited, which suggests an important protective role for catalase.

Key words:

lung diseases • TBA-reactive substances • lipid peroxidation • oxidative stress • antioxidants • alveolar epithelium • type II pneumocytes

Abbreviations:

AECs – alveolar epithelial cells, ARDS – acute (adult) respiratory syndrome, ATZ – aminotriazol, FBS – fetal bovine serum, PMNLs – polymorphonuclear leukocytes, SOD – superoxide dismutase, TBA – thiobarbituric acid, TBARS – thiobarbituric acid reactive substances, MTT – 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS – phosphate-buffered saline.

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INTRODUCTION

Type II pneumocytes make up a small percentage of the alveolar surface, but comprise 75% of the population of alveolar cells. The main functions of type II pneumocytes include surfactant production, xenobiotic metabolism, antioxidant protection, and electrolyte/fluid transport through the alveolus. They are progenitors of type I pneumocytes, and together with other cells compose the capillary-alveolar barrier^{4, 18, 24}. The outcome of lung injury may be influenced by the ability of a population of type II cells to restore the alveolar epithelium.

Oxidant-mediated type II cell damage may be one of the possible mechanisms resulting in cell death. It may lead to peroxidation of membrane lipids, DNA strand breaks, inactivation of enzymes and cell proteins, or inactivation of surfactant by peroxidation of its phospholipids and proteins^{1, 2, 7, 13, 15, 22, 28}.

Hydroxyl radical is one of most toxic reactive oxygen species in biological systems. It may be generated in the Fenton reaction involving hydrogen peroxide (H_2O_2) and ferrous ions (Fe^{2+}). H_2O_2 is a product of dismutation of the superoxide radical catalyzed by intracellular antioxidant, superoxide dismutase (SOD). Among the various mechanisms responsible for the clearance of H_2O_2 from a cell, the most important are those involving intracellular catalase and glutathione peroxidase. All the above antioxidants are complementary and are indispensable for protection. Simon et al.²⁶ showed *in vitro* that both the inhibition of catalase and glutathione peroxidase augmented cytotoxicity to rat type II alveolar epithelial cells (AECs) exposed to H_2O_2 , but only inhibition of catalase resulted in decreased H_2O_2 clearance. Transferrin-catalase conjugate showed excellent protection against oxidative injury and alteration of permeability in cultured alveolar epithelium²⁵. Serum from septic patients with acute respiratory distress syndrome (ARDS) had higher catalase activity and catalase-dependent H_2O_2 scavenging activity. On the other hand, the level of glutathione reduced form peroxidase activity was not different from that of septic patients without ARDS or of healthy controls¹⁴.

Catalase, although typically associated with peroxisomes, can also be detected outside these organelles. Its activity can be detected in acellular environments such as serum¹⁴ and bronchoalveolar lavage fluid²⁹, which suggests that the enzyme may be released from peroxisomes and further outside the cell, thus contributing to the antioxidant defense of cellular membranes.

The susceptibility of AECs to oxidant-mediated injury is unclear. According to many studies, type II

AECs are more resistant to oxidative stress than are endothelial cells and type I AECs^{3, 9, 17, 19, 27}. In view of the above, we wished to determine whether type II pneumocytes are susceptible to oxidative stress. As the cited data on catalase suggest an important role for this antioxidant, its role for the protection of AECs against oxidative stress has also been challenged.

MATERIALS AND METHODS

Reagents and materials

Trypsin, Percoll, fetal bovine serum (FBS), antibiotics, linoleic acid, aminotriazol (ATZ), and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (Germany). Waymouth medium was purchased from Gibco BRL (United Kingdom) and DNase-1 from Boehringer-Mannheim (Germany). DC Protein Assay (Bio-Rad, USA) was used for protein assessment. Culture grade plastic plates were purchased from Sarstedt (Germany). Pentobarbital (Vetbutal) was from Biowet-Pulawy (Poland). All other reagents used but not listed here were purchased from Sigma-Aldrich. All reagents used for cell isolation and maintenance in culture were of cell culture grade. Freshly prepared phosphate-buffered saline (PBS) was always used for preparing solutions of reagents, washing cells etc. It consisted of 133 mM NaCl, 1.89 mM $CaCl_2$, 5.2 mM KCl, 1.29 mM $MgSO_4$, 2.59 mM phosphate buffer pH 7.4, 10.3 mM HEPES buffer pH 7.4, and 1 mg/ml glucose. The same buffer was used throughout the experiment and is further called PBS solution.

Animals

Cells were isolated from male Wistar rats, 180–220 g body weight. The animals were bred in the animal house of the Medical University of Łódź. They were fed a normal diet and water. Experiments were performed according to local regulations concerning experiments on animals. All members of the research team directly involved in the experimental procedures with animals had an individual license to work with experimental animals issued by Local Ethics Committee for Experiments on Animals at the Medical University of Łódź.

Cell isolation and culture

The original protocol of the isolation of type II pneumocytes from rat was originally presented by Richards et al.²⁴ and also described in detail in our previous paper²¹. Briefly, animals were deeply anes-

thetized with an intraperitoneal injection of 50–100 mg pentobarbital. Lungs were dissected, lavaged with saline, and incubated with 0.25% trypsin in PBS solution (30 min, 37°C). Afterwards the lungs were chopped and the digestion was stopped with fetal bovine serum (FBS). The chopped lungs were suspended in DNase-1 solution (250 µg/ml), shaken, and filtered through nylon filters. The suspension was put on Percoll layers of heavy (1.089) and light (1.040) density. After centrifugation (20 min, 10°C, 250 × g) the layer containing type II cells was collected, suspended in DNA-se 1 solution (50 µg/ml), and centrifuged again (10 min, 10°C, 250 × g). The pellet was suspended in culture medium supplemented with L-glutamine and antibiotics (penicillin plus gentamycin) and incubated for 1 h on a plastic plate to decrease contamination with other cells (macrophages, fibroblasts). The purity of the final isolate was assessed by Papanicolaou stain. Type II pneumocytes comprised 87±8% of the final isolate. Cell viability estimated with trypan blue exclusion test was >90%. Cells were either suspended in PBS solution prepared as described in “Reagents and materials” and used for experiments as freshly isolated cells or cultured for 72 h on plastic culture grade plates in Waymouth medium containing antibiotics and L-glutamine and supplemented with 5% FBS. The medium was changed daily.

Generation of oxidants

Freshly isolated cells (5×10^5) suspended in PBS or 1×10^7 cultured cells were incubated for 30 min with 2 mM H_2O_2 and 20 nM $FeCl_2$. Their potential to induce lipid peroxidation was checked in a tube test with human erythrocytes and polymorphonuclear leukocytes (PMNLs). Under these conditions, thio-barbituric acid reactive substances (TBARS) in erythrocytes increased 2.5–7.0 nmol/g protein, and in PMNLs 1.1–5.6 nmol/g protein.

Protein assay

The DC Protein Assay of Bio-Rad was used. This assay is based on the Lowry method¹⁶.

Inhibition of intracellular catalase

Cells were pre-incubated for 30 min with 50 mM ATZ, a specific catalase inhibitor^{12, 26}.

Estimation of lipid peroxidation

After exposure to oxidants, fresh cells were centrifuged and suspended in PBS in portions of 5×10^5 . Cultured cells were scraped from the plastic plate

with a spatula (about 1×10^7 cells when seeded) and also suspended in PBS. The medium containing H_2O_2/Fe^{2+} was collected, centrifuged, and the pellet consisting of detached cells was added to the scraped cells. Before estimation of lipid peroxidation, both fresh and cultured cells were suspended in 0.1% Triton solution to obtain cell lysis. Concentration of TBARS was measured by the spectrophotometric method based on the reaction of lipid peroxides with TBA. Measurements were done at 532 nm, with Malonaldehyde bis (Bimethyl Acetal) as a standard. The lower detection limit was 5 nM. Results were expressed as nmol/g protein²³. All measurements were done 3 times.

Cytotoxicity

The MTT test was based on the reduction of MTT to formazan dye, a reaction catalyzed by mitochondrial succinic dehydrogenase. This enzyme belongs to the respiratory chain and is active only in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells⁵. Cells cultured for 72 h on 96-well plastic plates were rinsed twice with PBS and incubated for 1 h at 37°C with 0.5 mg MTT/1 ml PBS. Afterwards, the MTT solution was poured out and 100 µl DMSO was added. Finally the sample was shaken for 30 s before measurement. Freshly isolated cells in portions of 5×10^5 were incubated in Eppendorf tubes for 1 h at 37°C with 100 µl of MTT solution prepared as above. Then the cells were centrifuged (5 min, 6000 rpm, 10°C) and 100 µl DMSO was added. Measurements were done on a multiplate reader at 550 nm, with reference at 655 nm. Results were expressed as percent activity of untreated cells.

Statistical analysis

Data were expressed as mean ± SEM. The estimated distribution of data sets was not normal. A paired, non-parametric (Wilcoxon) test was used; $p \leq 0.05$ was considered significant.

RESULTS

Freshly isolated cells

Concentration of TBARS in unexposed cells was 4.0 ± 1.3 nmol/g protein and after exposure to H_2O_2/Fe^{2+} 8.3 ± 2.2 nmol/g protein ($n=6$, $p=0.0313$). This is shown in Fig. 1A. Inhibition of catalase in cells exposed to oxidants ($n=5$) resulted in an almost four-fold increase in TBARS: 4.5 ± 1.5 nmol/g protein for control (oxidant-exposed and ATZ – unexposed cells) and 16.2 ± 3.9 nmol/g protein in ATZ-exposed

cells (not statistically significant, $p=0.0625$; Fig. 2A). In cells pretreated with ATZ but not exposed to oxidants, the level of TBARS was not different from that observed for control cells, neither those exposed to ATZ nor to oxidants (6.85 ± 0.64 vs. 7.00 ± 0.49 , $n=3$; data not presented graphically).

The activity of mitochondrial succinic dehydrogenase in freshly isolated cells treated with H_2O_2/Fe^{2+} was not suppressed ($85\pm8.7\%$ of control, $n=3$). ATZ alone was toxic to cells and caused an almost total absence of enzyme activity ($9\pm4\%$ of control, $n=3$). These results are shown in Fig. 3A.

Cultured cells

Exposure to oxidants doubled the level of TBARS in cultured cells from 1.72 ± 0.39 (unexposed cells) to 4.36 ± 1.74 nmol/g protein ($n=4$, not significant, $p=0.125$; Fig. 1B). Inhibition of catalase in cells exposed to oxidants also caused an approximately 2-fold (although not significant) increase in TBARS concentration (4.04 ± 0.84 for matched oxidant-exposed cells and 7.55 ± 3.96 for ATZ-exposed cells, $n=3$; Fig. 2B). A negligible decrease was observed in mitochondrial succinic dehydrogenase activity in cells treated with H_2O_2/Fe^{2+} ($82\pm12.5\%$ of control, $n=3$). However, cultured cells treated with ATZ alone revealed remarkable cytotoxic effect ($27\pm17.5\%$ of control, $n=3$). See Fig. 3B for these results.

DISCUSSION

In the present study we show that exposure of alveolar cells to oxidants may cause an increase in the concentration of TBARS. As the most prominent component of TBARS is malonyldialdehyde, one of the low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products, the test is often used as a diagnostic index of lipid peroxidation¹⁰.

Although the increase in TBARS was significant only in freshly isolated cells, there was also a 4-fold increase in the mean value of TBARS in cultured cells. The lack of statistical significance even when the mean values of the test and control differ several times may have several reasons. The most important reason, however, is related to individual variability, even though the procedure was standardized and the animals were of the same strain. Due to the high number of cells necessary for each experiment, 4–6 animals were needed to obtain about 6×10^7 cells. In addition, each experiment was repeated 3–6 times. Some authors use cell lines in order to omit this problem; however, in the opinion of others, including the

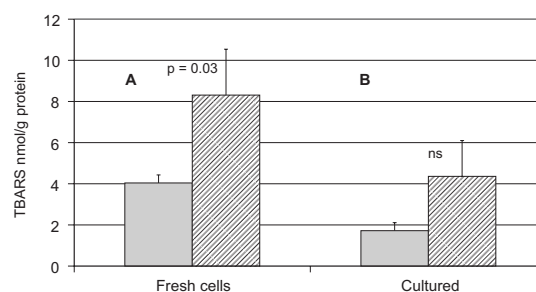


Figure 1. **A** – TBARS concentration (nmol/g protein) in freshly isolated cells. The shaded column shows results in unexposed cells. The striped one after exposure to H_2O_2/Fe^{2+} . Significant at $p=0.03$. **B** – TBARS concentration (nmol/g protein) in 72-h cultured cells in unexposed cells (shaded column) and after exposure to H_2O_2/Fe^{2+} (striped column). Abbreviation: ns – not significant. Data presented as mean $\pm$ SEM.

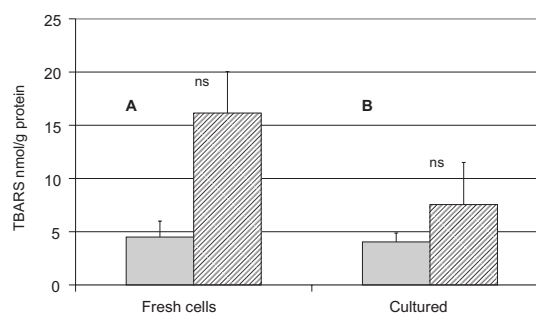


Figure 2. **A** – TBARS concentration (nmol/g protein) in freshly isolated cells exposed to H_2O_2/Fe^{2+} not preincubated with ATZ (control cells, shaded column) and cells preincubated with ATZ (striped column). **B** – TBARS concentration (nmol/g protein) in 72-h cultured cells exposed to H_2O_2/Fe^{2+} not preincubated with ATZ (control cells, shaded column) and cells preincubated with ATZ (striped column). Abbreviation: ns – not significant. Data presented as mean $\pm$ SEM.

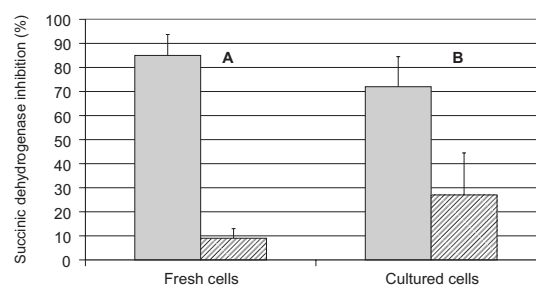


Figure 3. **A** – MTT test in freshly isolated cells. Succinic dehydrogenase activity expressed as percentage of control (of cells not preincubated with ATZ and not exposed to H_2O_2/Fe^{2+}) in cells exposed to H_2O_2/Fe^{2+} (shaded column) or exposed to ATZ (striped column). **B** – MTT test in cultured type II pneumocytes. Succinic dehydrogenase activity expressed as percentage of control (of cells not preincubated with ATZ and not exposed to H_2O_2/Fe^{2+}) in cells exposed to H_2O_2/Fe^{2+} (shaded column) or exposed to ATZ (striped column).

authors of the present study, this is an artificial model (cell lines originate from neoplastic cells which undergo several passages). Regardless of these

restrictions, our observation suggests that peroxidation of cellular lipids may also occur in alveolar cells *in vivo*. Inhibition of intracellular catalase further increases TBARS production, both in fresh and cultured cells. The most striking observation, however, is the lack of toxicity in cells with increased TBARS concentration. This may be related either to the non-specificity of the test or to the possibility that cellular membranes are not the predominant source of lipids.

It is generally believed that type I pneumocytes are more susceptible to oxidants than are type II cells^{3, 8, 20}. It was shown that type I cells possess low antioxidant enzyme activity. AECs that differentiate *in vitro* towards the type I morphology possess about 25–70% of the activities of SOD, catalase, and glutathione peroxidase, which were primarily measured in cells with the type II morphology^{8, 11}. The capacity of the cells to scavenge extracellular H₂O₂ was also decreased¹¹. In our previous study²¹ we found that the percentage of type II cells in prolonged culture was 93±4% on day 2, 73±14% on day 4, 56±15% on day 6, and 29±29% on day 8. These results indicate that cells cultured for 72 h under identical conditions should still contain over 70% of type II cells, and that the percentage of cells resembling type I cells exceeding 40% was observed only after 6 days in culture. However, the changes in cell morphology are not necessarily parallel to physiological changes. Dimova et al.⁶ showed that type II pneumocytes in culture rapidly lose most xenobiotic-metabolizing enzyme activities, despite preserving the type II morphology. In our already cited work²¹ we estimated the release of H₂O₂ and the influence of catalase inhibition on H₂O₂ release by type II pneumocytes in culture up to 8 days. The H₂O₂ concentration in response to ATZ increased significantly only at day 2, but insignificant increases were observed at each time point (measurements were done at 2-day intervals) up to the

final, 8th day of culture. In the present study we used ATZ in a concentration 50 mM. The same concentration was used by Simon et al.²⁶, who also estimated cytotoxicity of AECs exposed to H₂O₂. These authors did not observe significant toxicity of 50 mM ATZ (in cells not exposed to oxidants), but they agree that higher concentrations may be toxic. They also reported that the pure H₂O₂ exposure did not increase cytotoxicity, which increased considerably in ATZ-preincubated cells. These observations are consistent with ours, despite unexpected ATZ toxicity in both freshly isolated and cultured cells. The difference in toxicity threshold for ATZ may be related to slight differences in isolation technique and cell maintenance. On the other hand, the toxicity of ATZ may be strictly linked to its inhibitory action towards catalase, and because of that these results should not be disregarded. In our study, although the concentration of TBARS increased when oxidant-exposed cells were pre-incubated with ATZ (even though in neither fresh nor cultured cells did it reach the level of significance), freshly isolated cells not exposed to oxidants but treated with ATZ did not show increased peroxidation. Thus it seems unlikely that ATZ-induced cell toxicity is related to peroxidation of cellular membrane lipids.

In conclusion, our results indicate that type II AECs are not resistant to oxidative stress. We cannot, however, provide a clear explanation as to why cells with evidence of lipid peroxidation do not show increased cytotoxicity. Inhibition of catalase with ATZ, even in cells not exposed to oxidants, resulted in remarkable cytotoxicity. It seems unlikely that this effect is related to decreased antioxidant protection that could result in oxidative cell damage. However, in cells exposed to oxidants, lipid peroxidation products may further increase when intracellular catalase is inhibited. The latter would suggest that catalase is important in the antioxidant protection.

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