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Resistance of Quiescent and Proliferating Airway Epithelial Cells to H_2O_2 Challenge

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Running title:

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Abstract

Alveolar epithelial cell injury and recovery are important in the pathogenesis of oxidant-induced lung damage. The alveolar cell line A549 was used to study responses of proliferating and quiescent cells in culture to time and dose dependent hydrogen peroxide (H_2O_2) challenges.

Recovery was monitored after 24h of incubation in fresh medium with 10% serum. The adherent cells were counted and the resistance and recovery of the attached cells was assessed by appearance, by measuring the number of viable, apoptotic, and necrotic cells using Fluorescent Activated Cell Sorting (FACS), and by determining the intracellular free thiol content.

A549 cells recovered from a 1h challenge with up to 1mM H_2O_2 but could not sustain a more prolonged challenge (6 or 24h) with 0.5 mM or 1.0 mM H_2O_2. These more severe conditions resulted in loss of cells by detachment from the plate surface, reduced numbers of viable cells primarily due to necrosis and a strong reduction of the intracellular free thiol content.

Quiescent cells proved to be more sensitive to oxidative stress than proliferating cells. Intracellular free thiol levels apparently play a decisive role in cell survival, preferentially protecting proliferating cells.

Keywords

COPD; Epithelial cells; Apoptosis, Necrosis; Oxidative stress; Resistance; Thiols

Introduction

Oxidative stress on a cellular level is a combination of an increase in reactive oxygen species (ROS) exposure, a decrease in antioxidant protection, and a failure to repair oxidative

damage. Oxidative stress and the damage that may result from it have been implicated in a wide number of disease processes including inflammation, neuronal degeneration, and cancer [1;2].

Lungs are exposed to high levels of oxygen and in the case of smokers, high levels of radicals (10^{14} radicals per puff) are inhaled [3]. Chronic Obstructive Pulmonary Disease (COPD), which develops in 20% of smokers, encompasses both chronic bronchitis and emphysema, currently the fourth leading cause of death in the western world [4]. Emphysema due to oxidative stress and continuous inflammation are major hallmarks of COPD [5]. ROS, either directly from inhaled smoke and/or indirectly from inflammatory cells, may play a role in inflammation [6;7], through the formation of lipid peroxidation products, the activation and phosphorylation of mitogen-activated protein kinases (MAPKs), and through the activation of redox-sensitive transcription factors such as nuclear factor-kappa-ß and activator protein-1 [8].

Damage by ROS can induce apoptosis (programmed cell death) or necrosis of lung epithelial cells, which is followed by recovery and repair by proliferation of residual cells [9]. To obtain a detailed description of recovery of cells after an oxidative challenge, we investigated the response of the alveolar epithelial cell line A549 to various concentrations of H_2O_2. The effect of H_2O_2 on pulmonary epithelial cells has been studied in earlier investigations using different concentrations and different culture conditions [10-15]. However, the different abilities of proliferating and quiescent lung epithelial cells to recover from various concentrations of H_2O_2 have not been studied in a time-dependent manner. A549 is a adenocarcinoma cell line, but related to alveolar epithelial cells as earlier studies show [11;16], and is known to be sensitive to morphological changes under different stress conditions [17].

Inflamed lung tissue of smokers is exposed to high H_2O_2 concentrations. This is reflected in elevated H_2O_2 levels in exhaled breath condensate of smokers or patients with exacerbated COPD, compared to ex-smokers or non-smokers [18]. However, the level of H_2O_2 that is found in healthy or in inflamed lung epithelial cells is unknown. We hypothesize

that resistance and recovery will be dependent not only on the concentration of the oxidative agent but also on the duration of exposure and on the quiescent or proliferating state of airway epithelial cells. To address the hypothesis of this study we established conditions under which A549 cultures were quiescent to enable comparisons with proliferating cultures. To define concentrations of H_2O_2 that lead to survival or irreversible damage, and to determine whether these concentrations are different for quiescent versus proliferating cells, we assessed the morphology of the adherent cells, as well as their viability, apoptotic, and necrotic status.

Various studies describe H_2O_2 as an agent that may indirectly oxidize the free thiol groups (-SH) of cysteines in peptides and proteins within different types of human cells. In order to sustain their antioxidant defence, cells regenerate the oxidized peptides or proteins via enzymatic reduction and by increasing the expression of antioxidant peptides and proteins. Two major components of thiol reducing systems, glutathione (GSH) and thioredoxin (TRX), have been identified from various kinds of tissue and appear to dominate the cellular thiol redox potential [19;20]. We hypothesize that resistance and recovery of airway epithelial cells to H_2O_2 is determined by the pool of reduced thiol-components. Therefore, we monitored the effect of H_2O_2 on the redox state of airway epithelial cells by measuring the total free thiol content before and after a 24h recovery period for both proliferating and quiescent cells.

Materials and methods

Study design

Basal conditions (no H_2O_2) were used as controls for the 1, 6, and 24h of incubation of quiescent (non-dividing) and proliferating cultures with 0, 0.1, 0.5, and 1.0 mM H_2O_2, followed by a 24h recovery period (See Fig. 1A). The parameters that were studied to assess resistance and recovery of A549 cultures were cell morphology, number of adherent cells, cell viability, apoptosis, necrosis, and the intracellular free thiol content relative to the protein content (thiol redox state).

A549 epithelial cells

The human alveolar type II epithelium-like adherent cell line, A549 (ATCC number CCL-185), was maintained in continuous culture, split ratio 1:5, at 37°C, 5% CO_2 in RPMI-1640 with L-glutamine (Cambrex, Verviers, Belgium) 20 μg/mL gentamicin (Centafarm Services, Etten-Leur, The Netherlands) and 10% foetal calf serum (FCS; Cambrex, Verviers, Belgium). Polymerase chain reaction-tests (PCR) for mycoplasma were negative.

Cell proliferation

Proliferation of A549 cells was measured using a cell proliferation ELISA assay (Amersham, Diegem, Belgium). Briefly, 5000 cells were cultured in a 96-well microtitre plate at a final volume of 100 μl RPMI supplemented with 10% FCS and BrdU (10 μM) per well. After 24h the medium was removed. For quiescent cells, the cells were re-incubated overnight in serum free medium and BrdU. Proliferating cells were maintained in medium with 10% FCS and BrdU. BrdU incorporation was measured after 24h (both 10% FCS), 48h (with and without 10% FCS) and 72h (with and without 10% FCS) according to the manufacturer's instructions.

Challenge of proliferating or quiescent epithelial cells with H_2O_2

$0.8x10^5$ A549 cells per well were seeded in sterile 24-well culture dishes (Costar Europe Ltd, Badhoevedorp, The Netherlands) and incubated for 48h in the presence of serum. (proliferating cultures). For quiescent cultures, the medium with 10% FCS was removed after 24h of incubation and the cultures were incubated for another 24h in serum-free medium. 0.1, 0.5 and 1 mM H_2O_2 (Sigma, St Louis, MO, USA) was added to the cultures and incubated at 37°C for 1, 6, and 24h. After incubation, the medium with H_2O_2 was removed and the cells were either washed with sterile Phosphate Buffered Saline (PBS, Ca^{2+} and Mg^{2+} free, pH7.4) and analyzed or washed and incubated for a further 24h in fresh medium with 10% FCS (recovery phase). The remaining adherent cells were photographed using a Leica phase

contrast microscope (5x) and Leica digital camera to assess the morphology of the cells in the cultures.

Flow cytometry

After H_2O_2 challenge and the subsequent recovery phase, cells were washed and the adherent cells were detached by 5 minutes incubation with 0.05% trypsin in 0.53 mM EDTA (GIBCO, Invitrogen Company, Burlington, ON, Canada) at 37^{o}C, collected, centrifuged (300*g* for 5 min), and stained for Fluorescent Activated Cell Sorting (FACS) analysis of apoptosis (Annexin V-FITC) and necrosis (Propidium Iodide (PI)). The Annexin V–FITC/PI Apoptosis/Necrosis Detection Kit was used according to the manufacturer's instructions (Immune Quality Products (IQP), Groningen, The Netherlands). Cell-suspensions were analyzed on a FACS Calibur instrument with CellQuest software (Becton Dickinson, Heidelberg, Germany). Ten thousand events were collected for each sample and all analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregates. Unlabeled cells and labelled basal cultures were used to set the limit of the quadrants; the boundaries of the first quadrant (Annexin V^{low}-PI^{low}) were set to contain 100% of an unlabeled cell-suspension and 95% of the labelled cells of basal cultures. These settings were used to analyze all labelled cell-suspensions.

Total protein and free thiol measurements

Cells were washed and lysed by 1 freeze thaw cycle in 200 µl demineralised water and 50 µl were used for protein determination by the Bradford assay [21], using a bovine serum albumin (BSA, Sigma) standard curve. The samples were measured at 595 nm in a Biotek EL808 microplate reader (Beun de Ronde, Abcoude, The Netherlands). 12 mM 5,5‘-dithiobis-(2)-nitrobenzoic acid (DTNB, Sigma) in 0.1 mM potassium phosphate buffer, pH 7.0 (Merck, Haarlem, The Netherlands) was added to the rest of the lysed cells to a final concentration of 6 mM DTNB. After 10 minutes n the samples were measured at 405nm [22]. The amount of free thiols was calibrated against a standard curve of L-cysteine (Sigma).

Direct oxidation of BSA and L-cysteine

250 μM BSA in PBS containing 1 mM EDTA, or 250 μM L-cysteine in PBS, 1 mM EDTA, were incubated for 10 minutes in the dark with 0, 0.1, 0.5, and 1.0 mM H_2O_2 to assess the potential for direct oxidation. Before adding DTNB the excess H_2O_2 was inactivated with catalase. To 100 μl aliquots of the reaction, DTNB was added to a final concentration of 6 mM, and after 5 min incubation the thiol content was measured at 405 nm. The amount of free thiols was calibrated against a standard curve of L-cysteine.

Statistical analysis

The Student's *t*-test for paired observations was used for comparisons between the basal (non-stressed) values and the values directly after H_2O_2 incubation and recovery, and for the comparisons between proliferating and quiescent values. Data are expressed as average±SEM of 4 to 6 determinations per experiment. Each determination corresponded to one generation of A549. Differences were considered to be statistically significant when $p<0.01$.

Results

Proliferation and quiescence

The schematic outline of the experimental design is depicted in Figure 1A. The incorporation of BrdU was measured to determine the state of quiescence and proliferation. Fig. 1B shows that in the absence of serum, cultures become quiescent within 24 hours to remain quiescent during 72 hours.

Morphological analysis of A549 cells

The morphology of a cell is defined by its form and appearance, which can change in response to stimuli. Two clearly distinguishable morphologies could be discriminated (Fig. 2).

Morphology I (MI) relates to cells that are flat with a visible nucleus (Fig. 2A), where each cell has a diameter of approximately 20μm and is contacting neighbouring cells. Morphology II (MII) shows rounded cells that have shrunk in size to about 10μm in diameter (Fig. 2B) often losing contact with neighbouring cells. 95% +/- 3% of cells (proliferating and quiescent) in basal cultures at 80% confluence show morphology I (Fig. 2C). When cultures contained more than 15% rounded cells (Fig. 2D), the designation MII is added to Table 1, which summarizes the status of the cultures under various conditions of oxidative stress. When cultures contained more than 85% rounded cells (Fig. 2E) the cultures were considered to be MII, entirely.

Most cells in the cultures incubated for 1 hour with, 0.5mM, and 1.0mM H_2O_2 showed an overall morphology I indicative of healthy cells. As the time of incubation with 0.5 and 1.0 mM H_2O_2 increased from 1 to 6 and 24 hours, the overall culture changed from MI to MII. Once a culture contained more than 85% of MII cells, it could not recover in 24 hours to a MI culture. Judging by the number of cells that had changed to MII, quiescent cultures were slightly more sensitive to oxidative stress than proliferating cultures (Table 1).

Cell numbers and cell viability

In MII cultures, a part of the cells became detached from the cell culture dish. The severity of H_2O_2 stress was assessed by counting the cells that remained attached to the plate (Fig. 3), and analyse their viability by FACS (Fig 4). As a control for the cell counts (and the loss of cells), we measured the total protein content of basal and stressed cultures. The results of the protein assay corresponded to the cell counts (data not shown). FACS analysis showed that the number of viable cells in basal cultures and in the presence of H_2O_2 is generally lower in quiescent than in proliferating cultures.

After 1 hour of stress, the proliferating cultures showed a decrease of cell numbers as compared to the unstressed cultures, and an increase in apoptotic and necrotic cells. The quiescent cultures did not show this dose response after 1 hour, and contained even more viable cells after 1 hour of 1,0mM H_2O_2 challenge than the basal cultures. After 6 hours of

stress the cultures did not show a dose response as the number of cells remained constant, however the number of necrotic cells increased with increasing H_2O_2 concentration. This was more profound for quiescent cultures. During the 24 hour stress period a decrease of cell numbers corresponding to increasing H_2O_2 concentrations was observed, which was stronger for proliferating cultures than quiescent cultures. However, the residual adherent proliferating cells were more resilient to the challenge with 1.0mM H_2O_2, reflected by the higher number of viable cells than quiescent cultures.

After the recovery period, the basal cultures obviously had increased cell numbers. We observed that epithelial cells recovered from exposure to increasing H_2O_2 concentrations after 1 hour of incubation, as was shown by the increased viability of the remaining adherent cells. Similarly, epithelial cells recovered from 0.1mM H_2O_2 independent of exposure time. However, cell numbers did not reach the 'recovered' unstressed culture values (except the 1 hour incubation of proliferating cells).

During the recovery period after 6 hours of stress, the cell numbers had decreased, reflected by an increase of mainly necrotic cells. This increase in necrotic cells was more profound for quiescent cultures, especially after recovery from 0.5mM H_2O_2. After recovery from 24 hours of stress the cell numbers had increased slightly after 0.5mM H_2O_2 and had decreased slightly after 1.0mM H_2O_2.

The difference between proliferating and quiescent cultures becomes clear when analysing cell viability. The relative number of viable and necrotic cells did not change in proliferating cultures during the recovery period. However, in quiescent cultures after recovery from 1.0mM H_2O_2 the remaining adherent cells were significantly more viable than directly after the stress.

Oxidation of free (cellular) thiol groups

H_2O_2 is considered to be a precursor for radicals generated by mitochondrial metabolism. However, incubation of H_2O_2 in the presence of the metal chelator EDTA, and in the absence of light, showed a rapid oxidation of cysteines (Fig. 7).

The ratio between oxidized and reduced thiol groups can be considered as an indicator of oxidation. Since free thiol groups play an important role in the defence against oxidative stress, we measured the effect of H_2O_2 on the total cellular level of free thiols relative to the protein content (Fig. 5). Cellular Oxidative stress by H_2O_2 is reflected in a lower free thiol to protein ratio of most cultures, compared to basal unstressed values. First of all we observed that basal quiescent cultures have a higher thiol to protein ratio than proliferating basal cultures. However, the free thiol to protein ratio in quiescent cultures had diminished more strongly compared to the basal values especially after 24h of stress, opposed to the proliferating cultures.

After the recovery period, the quiescent cultures had returned to a proliferating state, and subsequently showed lower free thiol to protein ratios. Based on these changed basal values, the quiescent cultures subjected to 0.1mM H_2O_2 had recovered. At higher stress levels (0.5mM and 1.0mM) recovery of the thiol to protein ratio was still observed after 1h of stress and, remarkably, even after 24h of 0.5 mM H_2O_2.

After the recovery period of proliferating cultures, it was visible that the cultures recovered from all H_2O_2 concentrations for 1h of stress and from 0.1mM H_2O_2 for 6 and 24h. Remarkably, proliferating cultures showed a high free thiol to protein ratio after recovery from 6h and 24 h of 0.5mM H_2O_2. Another remarkable result was the presence of free thiols after recovery from 24h of 1.0mM H_2O_2 in proliferating cultures, whereas in quiescent cultures these values had diminished to zero. This indicates that some of the remaining attached cells of MII morphology (mainly necrotic cells), had responded to the challenge by strongly increasing their thiol redox balance. By measuring the total GSH content (GSH + GSSG), similar results were obtained (data not shown).

Cells entering necrosis have a low free thiol:protein ratio. Figure 6 relates the average thiol concentration in nmoles per mL to the percentage of necrotic cells. As the morphology is a visual indication of the stress level, Figure 6 also includes the different morphologies. In general, we observed a trend that cultures with a low thiol concentration contained the most MII cells and necrotic cells.

Discussion

H_2O_2 is one of the oxidants formed through oxygen metabolism and during inflammation as part of the oxidative burst. In studies on A549 and other cells, widely different conditions for H_2O_2 incubations were used [10-13;15] prompting us to perform a detailed time-dependent dose-response study and to define limits for *in vitro* resistance and recovery. For this purpose cells were challenged with H_2O_2 at concentrations above levels implicated in cell signalling (50 μM) [23], but below levels that would lead to cell death within minutes (10 mM). In most described cases A549 cells are maintained in medium containing serum up to the moment the experiment starts [11;12;15]. When the cultures are not 100% confluent it is likely that they are still proliferating at the start of the experiment.

In our present report we demonstrate that differences in cell viability and the relative content of free thiols of basal quiescent and proliferating cultures influence their ability to recover from oxidative stress. FACS analysis (Fig. 4) showed that a higher percentage of cells are viable in basal proliferating cultures compared to quiescent cultures. This indicates that cells are in a more viable state when they are growing in a medium containing 10% serum.

In contrast, basal quiescent cultures maintained a higher protein to thiol ratio (Fig. 5). These basal quiescent cultures showed a remarkable decrease in free thiols after the addition of serum, indicating that in general the thiol redox balance of proliferating cells is lower. It is important to note that our observations argue against a simple correlation between the free thiol:protein ratio and anti-oxidant defence during stress-free conditions. Quiescent cells have a higher thiol redox balance than proliferating cells but are nevertheless more sensitive to oxidative stress. Differences are most prominent after 6 and 24 hours exposures, especially to 0.5 mM H_2O_2 and subsequent recovery, where we observed that quiescent cultures are more vulnerable (Figs. 3, 4 and 5).

When the proliferating and quiescent cultures were stressed with H_2O_2 and subsequently incubated for recovery, we observed morphological differences and changes in cell numbers. These differences prompted us to investigate whether they could be related to a different physiology of quiescent cultures opposed to proliferating cultures. Furthermore, we found a causal relation between cell morphology, free thiol content and the number of necrotic cells for proliferating cultures and that this correlation is less strong for quiescent cultures (Fig. 6). These differences were not influenced by the thiol content of the external medium. We measured the thiol content of the medium prior to the addition of H_2O_2 and found that the thiol content is not significantly different in the medium of proliferating cultures compared to that of quiescent cultures (data not shown).

Our data aid in defining a threshold of H_2O_2 stress up to which A549 cells can recover when supplemented with serum-containing medium. All cultures could recover from short-term (1h) exposure to 0.1 - 1.0 mM H_2O_2, or from long-term exposure (up to 24h) to low H_2O_2 (0.1 mM) concentrations. However, cells underwent irreversible changes after longer incubation times as indicated by a modified morphology (cell shrinkage) decreased cell viability, and an increased number of cells detaching from the plate surface, which is consistent with previous findings in literature [14]. This recovery from oxidative stress may mimic to some extent the situation *in vivo*, where epithelial cells may be exposed to oxidative stress followed by a recovery period (e.g. after a smoking episode).

It is of interest to study how both quiescent and proliferating epithelial cells cope with oxidative stress, as there are indications that cells start to proliferate upon lung damage. When the lungs are damaged, both quiescent and proliferating cells are likely to respond differently to oxidative stress. It has been reviewed that upon tissue damage, growth factors are responsible for proliferation and remodelling of airway epithelium to repair the damage in COPD [24]. However, solid data on the *in vivo* rate of proliferation in the human lung is not available. An indication of the proliferation-rate is derived from literature on pulmonary gene therapy studies, where 28 days after transient transfection with an adenoviral vector the expressed transfected gene product had disappeared in the mouse lung [25]. In current

experiments described in the literature, it is not always clear whether the cells were incubated with or without serum previous to the experiment.

In vitro quiescent cultures form a homogenous population of cells in the same state, facilitating analysis. Mammalian cells deprived of serum stop proliferating and become arrested, usually between mitosis and S-phase, in a specialized, non-proliferating G0 state, called quiescence. It is known that the cellular redox potential varies during the life span of a cell. For example, the cellular redox state has characteristic set points depending on whether cells are quiescent, proliferating, differentiating, or apoptotic [26;27]. We hypothesize that these differences explain in part the sensitivity of quiescent cultures to oxidative stress. The proliferating cultures are heterogeneous as cells are in different phases of mitosis, all responding more or less strongly to oxidative stress. Whereas the cells in the homogenous quiescent cultures will all respond similarly, marking the sensitivity and the lack of recovery of the cultures after 6h of 0.5 mM H_2O_2.

The lung epithelium *in vivo* is constantly exposed to high concentrations of oxygen and other oxidants from endogenous and exogenous sources, especially during smoking [28]. ROS causes cellular injury via reactions leading to more reactive species such as hydroxyl radicals and lipid peroxidation products. A source of ROS is H_2O_2, which is known to cause oxidative stress by depletion of free thiols. H_2O_2 is directly generated during cigarette smoking (~22 μg to 37 μg H_2O_2 per aqueous tar extract from 1 cigarette) [29]. Additionally, H_2O_2 is produced within epithelial cells as a result of mitochondrial metabolism, NADPH oxidase [14], glycollate and monoamine oxidase activity [23], and by superoxide (O2. −) dismutase in the cytoplasm. In this report we show that H_2O_2 is able to directly oxidize free thiols without the need for cellular metabolism as exemplified by the *in vitro* oxidation of BSA and L-cysteine (Fig. 7).

The fact that in our studies A549 cells went into a state of necrosis without any pronounced signs of apoptosis triggered us to study the activation state of Caspase-3, a cysteine proteinase, during a selected number of experimental conditions. Interestingly, there was no indication of Caspase-3 activation at 0.5 or 1 mM H_2O_2 after long-term incubation, as

studied by Western blotting (data provided by Dr. D.J. Slebos, not shown), which may explain the lack of apoptosis. Although not proven by us, it is likely that the active-site cysteine in Caspase-3 was directly oxidized by H_2O_2 or its metabolites thus inactivating the enzyme [30;31]. This is in line with out observations that H_2O_2 can directly oxidize free thiol groups e.g. in BSA and cysteine.

In conclusion, quiescent cultures appear to be more sensitive than proliferating cultures, especially to prolonged incubations with higher H_2O_2 concentrations. Our aim was to define the upper concentration limit of H_2O_2 from which quiescent and proliferating cells can recover when returned to serum-containing medium, in light of future studies on the analysis of proteins involved in the recovery process. This upper concentration limit appears to be 0.1 mM H_2O_2 as all cultures were able to recover from this concentration. Another important factor is time of exposure, as incubations with 0.5 and 1.0 mM H_2O_2 for longer than 1 hour resulted in loss of attached cells and an increase in necrotic cells. In proliferating cultures there are a limited number of cells remaining after severe oxidative stress and it appears that they have adapted to this condition by increasing their thiol:protein ratio considerably after recovery.

Many regulatory proteins contain critical cysteine residues that are sensitive to oxidation to sulfenic acids, forming of intra- and inter-molecular disulfides or mixed disulfides with glutathione (GSH) [32]. Free (reduced) thiol groups in general play an important role in the defence against oxidative stress and healthy cells are generally found in a reduced state having a large excess of reduced over oxidized thiols. The maintenance of this state is critical and dependent on the equilibrium between oxidized and reduced thiols. Currently we study how the free thiols in proteins of cultures react to these borderline conditions of oxidative stress in order to elucidate protective mechanisms that aid in stress resistance and to correlate this to recovery or cell death. These studies may elucidate the inter-individual differences in susceptibility to oxidation-mediated tissue damage and inflammation as is observed in 20% of smokers that develop COPD, whereas the remaining 80% do not show pulmonary limitations.

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References

1. Floyd RA. Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB J* 1990: 4: 2587-2597.

2. Finkel T. Oxidant signals and oxidative stress. *Curr Opin Cell Biol* 2003: 15: 247-254.

3. Pryor WA. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ Health Perspect* 1997: 105 Suppl 4: 875-882.

4. Viegi G, Scognamiglio A, Baldacci S, Pistelli F and Carrozzi L. Epidemiology of chronic obstructive pulmonary disease (COPD). *Respiration* 2001: 68: 4-19.

5. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, Der Mark TW, Koeter GH and Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000: 55: 12-18.

6. Maestrelli P, Saetta M, Mapp CE and Fabbri LM. Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001: 164: S76-S80.

7. Boots AW, Haenen GR and Bast A. Oxidant metabolism in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2003: 46: 14s-27s.

8. Rahman I. Oxidative stress in pathogenesis of chronic obstructive pulmonary disease: cellular and molecular mechanisms. *Cell Biochem Biophys* 2005: 43: 167-188.

9. Rutgers SR, Timens W, Kauffman HF and Postma DS. Markers of active airway inflammation and remodelling in chronic obstructive pulmonary disease. *Clin Exp Allergy* 2001: 31: 193-205.

10. Rahman I, Li XY, Donaldson K, Harrison DJ and MacNee W. Glutathione homeostasis in alveolar epithelial cells in vitro and lung in vivo under oxidative stress. *Am J Physiol* 1995: 269: L285-L292.

11. Anseth JW, Goffin AJ, Fuller GG, Ghio AJ, Kao PN and Upadhyay D. Lung surfactant gelation induced by epithelial cells exposed to air pollution or oxidative stress. *Am J Respir Cell Mol Biol* 2005: 33: 161-168.

12. Rahman I, Mulier B, Gilmour PS, Watchorn T, Donaldson K, Jeffery PK and MacNee W. Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kappaB. *Biochem Pharmacol* 2001: 62: 787-794.

13. Rahman I, Gilmour PS, Jimenez LA and MacNee W. Oxidative stress and TNF-alpha induce histone acetylation and NF-kappaB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 2002: 234-235: 239-248.

14. Dandrea T, Hellmold H, Jonsson C, Zhivotovsky B, Hofer T, Warngard L and Cotgreave I. The transcriptosomal response of human A549 lung cells to a hydrogen peroxide-generating system: relationship to DNA damage, cell cycle arrest, and caspase activation. *Free Radic Biol Med* 2004: 36: 881-896.

15. Lehtonen ST, Markkanen PM, Peltoniemi M, Kang SW and Kinnula VL. Variable overoxidation of peroxiredoxins in human lung cells in severe oxidative stress. *Am J Physiol Lung Cell Mol Physiol* 2005: 288: L997-1001.

16. Lannan S, Donaldson K, Brown D and MacNee W. Effect of cigarette smoke and its condensates on alveolar epithelial cell injury in vitro. *Am J Physiol* 1994: 266: L92-100.

17. Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ and Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J Allergy Clin Immunol* 2000: 105: 1185-1193.

18. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL and Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996: 154: 813-816.

19. Arner ES and Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 2000: 267: 6102-6109.

20. Rahman I and MacNee W. Lung glutathione and oxidative stress: implications in cigarette smoke- induced airway disease. *Am J Physiol* 1999: 277: L1067-L1088.

21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976: 72: 248-254.

22. DEAKIN H, ORD MG and STOCKEN LA. 'Glucose 6-Phosphate-Dehydrogenase' activity and thiol content of thymus nuclei from control and X-irradiated rats. *Biochem J* 1963: 89: 296-304.

23. Halliwell B, Clement MV and Long LH. Hydrogen peroxide in the human body. *FEBS Lett* 2000: 486: 10-13.

24. Chung KF. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 2001: 34: 50s-59s.

25. Koehler DR, Sajjan U, Chow YH, Martin B, Kent G, Tanswell AK, McKerlie C, Forstner JF and Hu J. Protection of Cftr knockout mice from acute lung infection by a helper-dependent adenoviral vector expressing Cftr in airway epithelia. *Proc Natl Acad Sci U S A* 2003: 100: 15364-15369.

26. Shackelford RE, Kaufmann WK and Paules RS. Oxidative stress and cell cycle checkpoint function. *Free Radic Biol Med* 2000: 28: 1387-1404.

27. Schafer FQ and Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001: 30: 1191-1212.

28. Cross CE, van d, V, O'Neill CA, Louie S and Halliwell B. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994: 102 Suppl 10: 185-191.

29. Yan F, Williams S, Griffin GD, Jagannathan R, Plunkett SE, Shafer KH and Vo-Dinh T. Near-real-time determination of hydrogen peroxide generated from cigarette smoke. *J Environ Monit* 2005: 7: 681-687.

30. Hampton MB, Stamenkovic I and Winterbourn CC. Interaction with substrate sensitises caspase-3 to inactivation by hydrogen peroxide. *FEBS Lett* 2002: 517: 229-232.

31. Baker A, Santos BD and Powis G. Redox control of caspase-3 activity by thioredoxin and other reduced proteins. *Biochem Biophys Res Commun* 2000: 268: 78-81.

32. Fratelli M, Demol H, Puype M, Casagrande S, Eberini I, Salmona M, Bonetto V, Mengozzi M, Duffieux F, Miclet E, Bachi A, Vandekerckhove J, Gianazza E and Ghezzi P. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci U S A* 2002: 99: 3505-3510.

Footnotes

(from table 1)

* proliferating cultures

† quiescent cultures

‡ both morphologies can exist in one culture, which is indicated in this table when one morphology exceeds 15% of the total cell number.

Table

Table 1. Morphology of proliferating and quiescent 75-85% confluent A549 cultures after exposure to H_2O_2 and subsequent recovery, based on two different appearances (Morphologies I and II, see Fig. 2). Distinct morphologies are: large, flat cells with clear nuclei (I) and small, rounded cells (II).

H_2O_2 incubation	1 hour		6 hours		24 hours	
	pro*	qui†	pro	qui	pro	qui
Basal morphology; no H_2O_2	I	I	I	I	I	I
After 24 h incubation; 10% serum	I	I	I	I	I	I
0.1 mM H_2O_2; after stress	I	I	I	I, II‡	I	I,II
0.1 mM H_2O_2; after recovery	I	I	I	I	I	I,II
0.5 mM H_2O_2; after stress	I	I, II	I, II	I, II	II	II
0.5 mM H_2O_2; after recovery	I	I	II	II	II	II
1.0 mM H_2O_2; after stress	I	I, II	I, II	II	II	II
1.0 mM H_2O_2; after recovery	I	I	II	II	II	II

* proliferating cultures

† quiescent cultures

‡ both morphologies can exist in one culture, which is indicated in this table when one morphology exceeds 15% of the total cell number.

Figure legends

Figure 1A. Experimental design to study the effect of H_2O_2 on proliferating and quiescent A549 cells. After 24 hours of proliferation, the cultures were made quiescent by replacing the medium with serum free medium and incubate for another 24 h. At time point '48', H_2O_2 (0.1, 0.5 or 1 mM) was added to the cultures and the cells were exposed to this oxidative stress for 1, 6 or 24 h. Exposure to H_2O_2 was stopped by either harvesting the cells for analysis or by replacing the medium containing H_2O_2 with fresh medium supplemented with 10% FCS (exemplified as time point '72') Cultures were allowed to recover for another 24 h before analysis ('end'). Proliferating cell cultures were maintained in medium containing 10% foetal calf serum (FCS) throughout the entire duration of the experiment. Control cultures were treated identically but without H_2O_2.

Medium with 10% serum (light grey), serum free medium (dark grey), incubation with 0.1, 0.5, or 1.0 mM H_2O_2 for 1, 6, or 24 h (white).

B. Determination of the quiescent and proliferating state of A549 cell cultures by measuring the incorporation of BrdU. After 24h in serum-free medium, the cultures were non-dividing (quiescent) (white bars) throughout the duration of the oxidative stress period (until 72 hours), while the cultures in serum-containing medium continued to proliferate. *** significant different population, $p<0.001$, n=4, students t-test, ns = non-significant

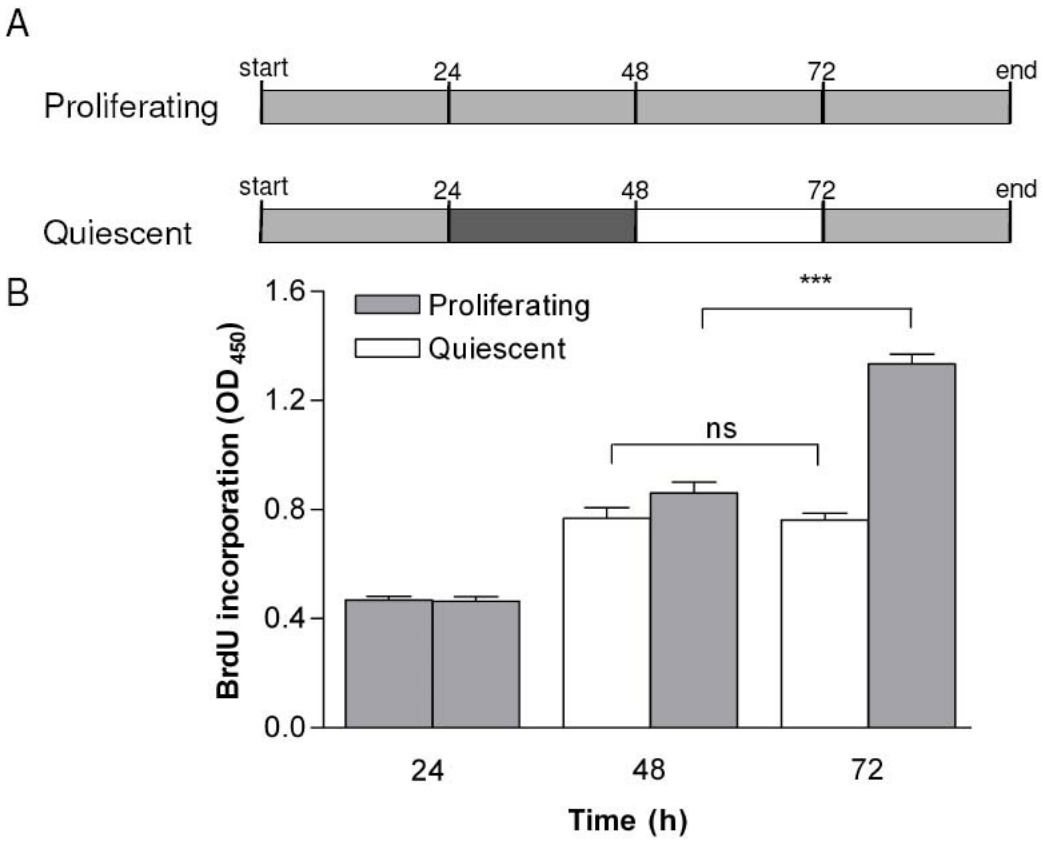


Figure 2. Observed morphological changes in A549 cells exposed to H_2O_2-mediated oxidative stress. Morphology I (A) shows cells that are flat, on average 20 μm in size, and have cell-cell contact. Morphology II (B) shows rounded cells that have shrunk in size to about 10 μm in diameter due to oxidative stress. Most cells have lost contact with neighbouring cells. Basal, unstressed cells are mainly of morphology I but contain 5% +/- 3% cells of morphology II (C). Both morphologies can occur in a single culture (D), as shown for quiescent cells that were exposed to 0.1 mM H_2O_2 for 6 h. Under severe stress situations (24 hours of 0.5 mM H_2O_2) all remaining cells are of morphology II (D). Scale bar: 10 μm.

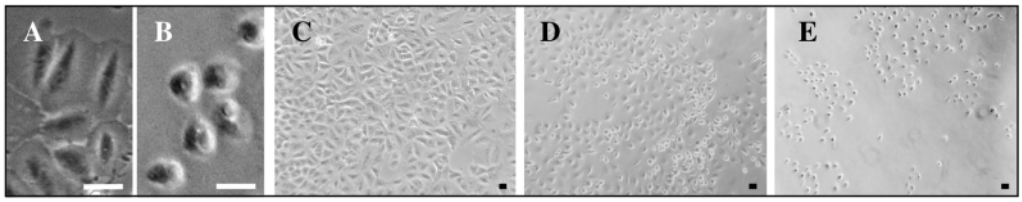


Figure 3. Effect of oxidative stress on cell numbers of proliferating and quiescent A549 cultures. Proliferating and quiescent A549 cell cultures were analyzed after 1 h, 6 h, and 24 h of incubation with 0 (basal), 0.1, 0.5 and 1.0 mM H_2O_2 (white columns) and after 24 h of recovery in the presence of serum-containing medium (black columns). '*' = $p<0.01$ difference of stressed samples versus basal cell numbers, 'o' = $p<0.01$ difference of cultures 'after recovery' versus cell numbers directly 'after incubation', n=4.

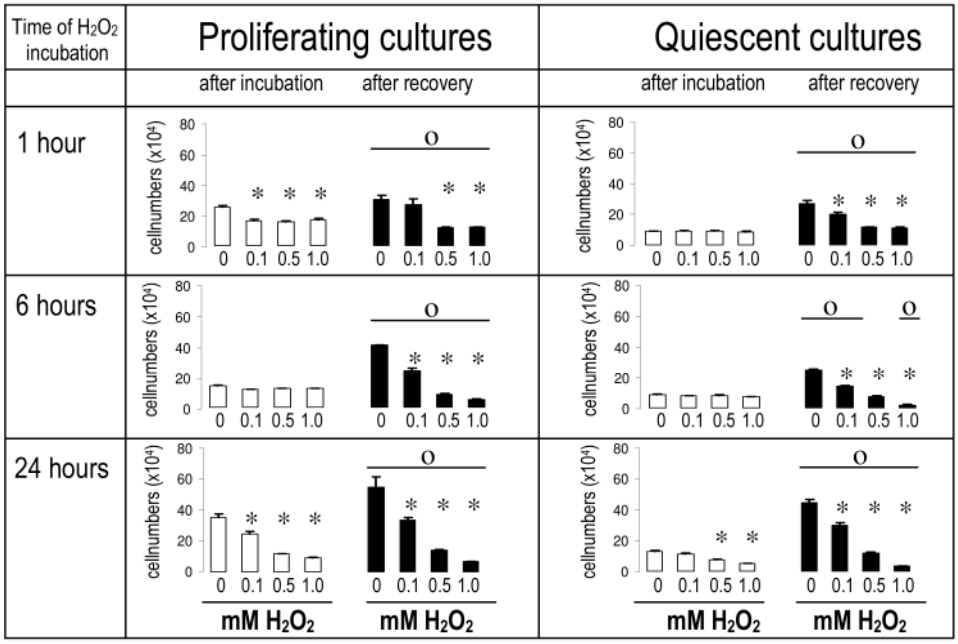


Figure 4. Percentage of viable, apoptotic and necrotic cells of proliferating and quiescent A549 cultures with H_2O_2 at various concentrations for increasing time periods. Cells with polarised, intact membranes (Annexin^{low}-PI^{low}) are considered viable (white bars). Cells with distorted membranes (Annexin^{high}-PI^{low}) are considered early apoptotic (grey bars). All cells with damaged membranes (Annexin^{low}-PI^{high} and Annexin^{high}-PI^{high}) are considered to be necrotic cells (black bars). 10^4 cells were counted per culture directly after incubation with H_2O_2 or after a 24 h recovery period. '*' = $p<0.01$ difference of viable cells in stressed cultures versus basal viable cultures, 'o' = $p<0.01$ difference of cultures 'after recovery' versus viable cells in cultures directly 'after incubation', n=5.

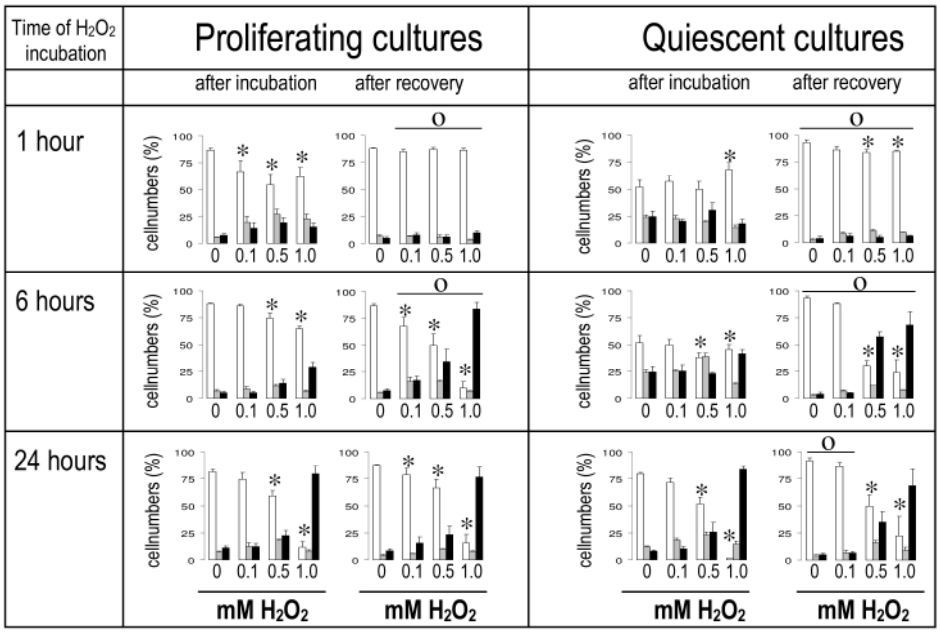


Figure 5. Oxidation of free thiols expressed as thiol to protein ratio (nmoles/mg) in A549 cells after exposure to H_2O_2 at various concentrations for increasing time periods.. Proliferating and quiescent cell cultures were incubated with 0, 0.1, 0.5, and 1.0 mM H_2O_2 for 1 h, 6 h and 24 h. The free thiol redox balance was calculated

directly after challenge (white bars) and after recovery (black bars). '*' = $p<0.01$ difference of stressed cultures versus the basal cultures, 'o' = $p<0.01$ difference of cultures 'after recovery' versus the thiol redox balance directly 'after incubation', n=4.

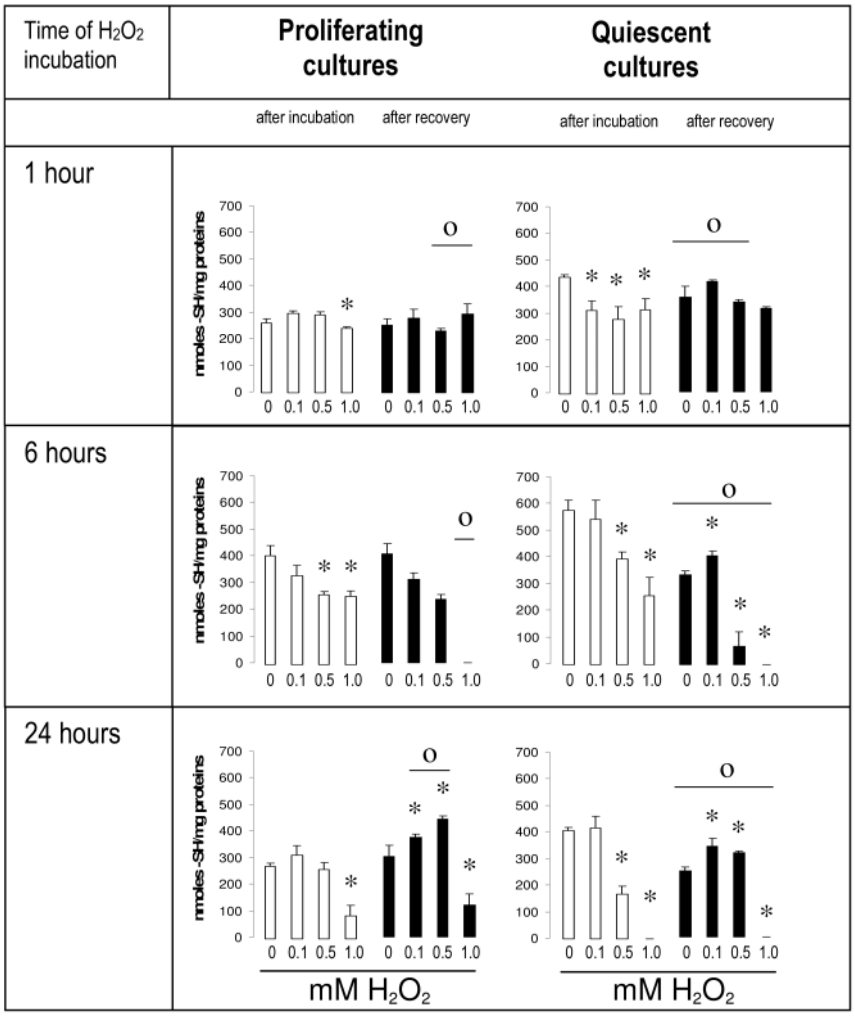


Figure 6. Correlation between the number of necrotic cells and the SH content of A549 cultures. Proliferating (A) and quiescent cultures (B) were analysed for free thiol content and percentage of necrotic cells after H_2O_2 administration and after a recovery period. All cells with damaged membranes (Annexin^{low}-PI^{high} and

Annexin^{high}-PI^{high}) are considered to be necrotic cells. The morphology of the cells in proliferating and quiescent cultures is shown: morphology I (white), cultures of mixed morphologies I and II (grey) and cultures with cells of morphology II (black) (Table1).

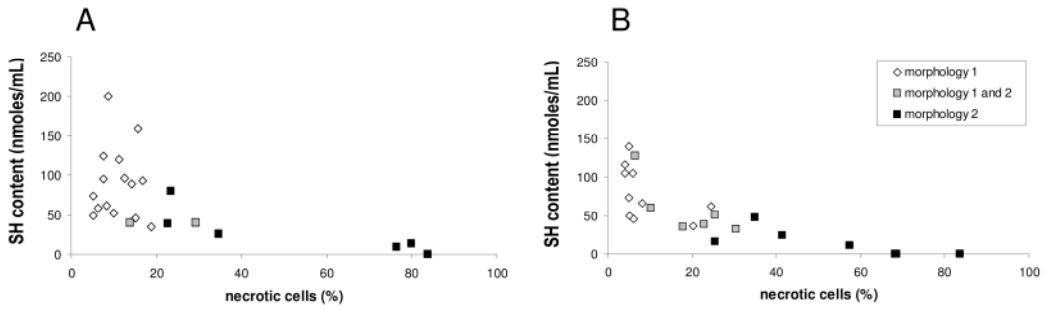
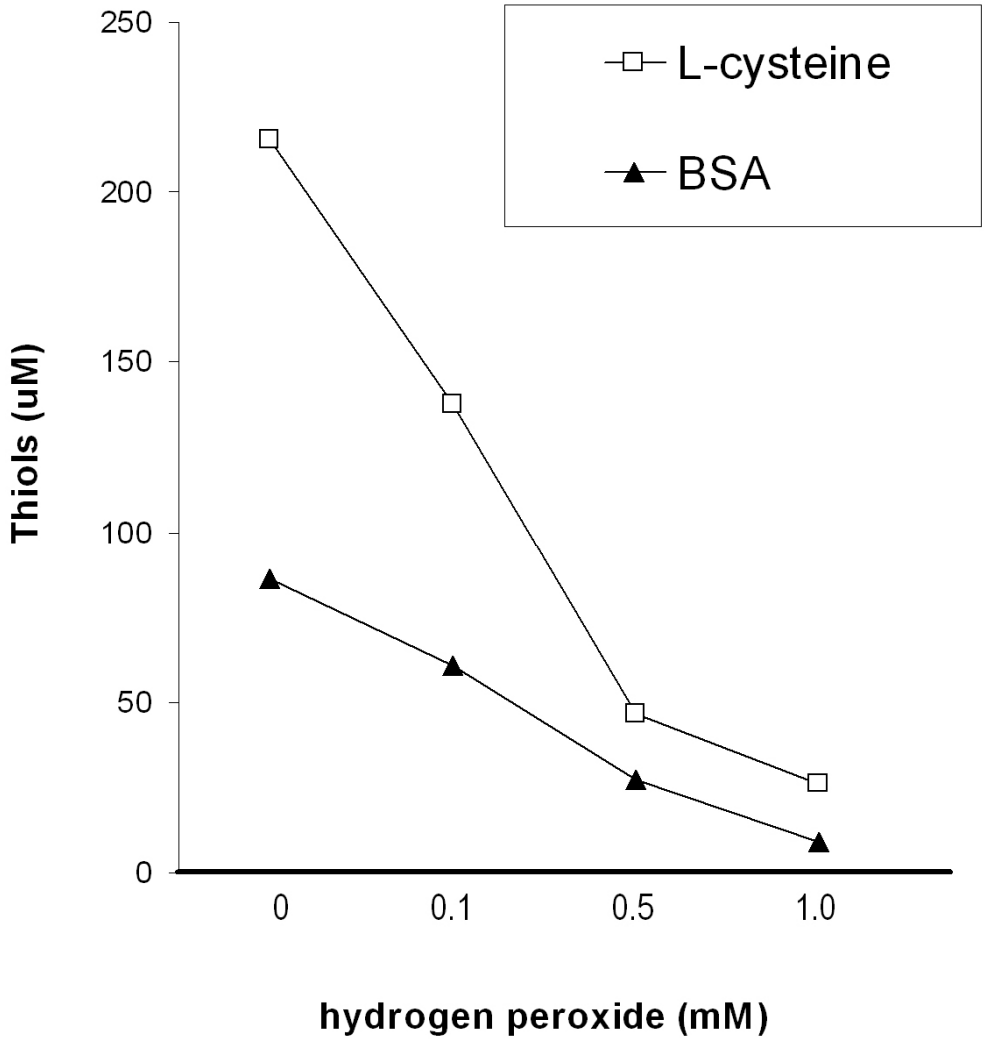


Figure 7. Direct oxidation of free SH groups by H_2O_2. The effect of 15 minutes incubation of 250 μM of BSA and L-cysteine with increasing concentrations of H_2O_2 in the absence of UV light and in the presence of EDTA.


250
200
150
100
50
0
Thiols (uM)
L-cysteine
BSA
0
0.1
0.5
1.0
hydrogen peroxide (mM)