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Pro-oxidative toxicity of cells in suspension does not point to a cell cultural artefact as an explanation of the increased susceptibility of alveolar epithelial-like cells after glucocorticoid pretreatment

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ABSTRACT

The influence of cell numbers on peroxide-(tertiary butylhydroperoxide (tBHP) or hydrogen peroxide (HP)) or zinc-(zinc chloride) induced oxidative stress was assessed in alveolar epithelial-like cell lines in this work. Differences in cell numbers change the cellular glutathione and glutathione reductase activity as well as the amount of exported glutathione and therefore might influence susceptibility against oxidative stress.

Toxicity due to zinc decreased, toxicity due to HP increased, while tBHP-mediated toxicity was unchanged in our experiments when cells were exposed in suspension as compared to monolayers. Toxicity of HP correlated to the glutathione content in monolayers and in cell suspensions, while zinc- or tBHP-mediated toxicity did not correlate towards glutathione.

Decreasing cellular glutathione and the activity of some antioxidative enzymes by glucocorticoid pretreatment had no effect on toxicity of zinc or tBHP in L2 cells in suspensions, while toxicity in monolayers was increased. Glucocorticoid pretreatment seems to increase toxicity of HP in A549 monolayers according to the lowered protein content, while toxicity might be changed by a different way when cells are incubated as cell suspensions. No explanation as a cell culture artificial effect was observed, therefore we assume the increased toxicity after glucocorticoid pretreatment occurs in vivo as well.

1. Introduction

Various pulmonary disorders, pathophysiologically based on oxidative stress, are treated with glucocorticoids (GC). Such disorders include e.g. asthma bronchiale, but administration of GC even is actually discussed in cases of an acute respiratory distress syndrome (Annane, 2007; Khilnani and Hadda, 2011; Okamoto, 2006; Thompson, 2010). In previous experiments an increase in zinc-mediated oxidative stress was found in cell culture experiments after pretreatment of alveolar epithelial type II-like cells with hydrocortisone (HC) by our group (Walther et al., 1999). This effect has been characterised as glucocorticoid-derived (Walther, 2004) and it was recently confirmed in our group for tBHP and HP with dexamethasone (DEX) as well (Walther and Mückter, 2009). Therefore pro-oxidative substances with glutathione-dependent and non glutathione-dependent mechanisms of detoxification are affected by glucocorticoids.

A critical appraisal of results derived from in vitro models should be common before transferring them to the in vivo situation. Therefore we questioned whether the increased toxicity of zinc in GC pretreated alveolar epithelial cells might be relevant for this cell type in vivo as well. A well known effect of GC is their katabolic action accompanied by a diminished cellular proliferation. Correspondingly, after 72 h of pre-treatment with GC, alveolar epithelial L2 or A549 cell layers regularly have decreased protein

Abbreviations: BSO, buthionine sulfoximine; CYS, L-cysteine; DEX, dexamethasone; DMEMmF12, Dulbecco's modified Eagle medium/Ham's F12 nutrient mix (1:1); DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); EDTA, N,N,N′,N′-ethylendiaminetetraacetate; Fcs, fetal calf serum; GC, glucocorticoid(s); GR, GSSG reductase, glutathione reductase; GSH, glutathione, reduced form; GSSG, glutathione, oxidized form; HBSS, Hanks' balanced salt solution; HC, hydrocortisone; HP, hydrogen peroxide; MEM, minimum essential medium; MET, L-methionine; MII, methionine incorporation inhibition; NADPH, nicotinamide-adenine dinucleotide phosphate, reduced; PBS, phosphate-buffered saline; SDS, sodium laurylsulfate; tBHP, tertiary butylhydroperoxide.

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contents (including GR, and GSH as well) (Rahman et al., 1998; Walther, 2004) and a lower number of cells as compared to non-pretreated cell layers. The question arises whether the increase in oxidative toxicity is caused by an unspecific, e.g. on total content of proteins based effect (this might be caused by an unspecific protein-affinity and might be a hint for a cell culture artificial effect). Alternatively, with the cellular changes due to GC an increase in the volume of the single cells seems to occur, therefore a changed "incorporation kinetic" might occur. A further explanation might be that an unusual cell number dependent toxicity exists. This latter explanation might be based on a single (or various) limiting enzyme activity(s). Especially in the case of zinc the strong affinity towards proteins can influence the intensity of toxic effects. Nevertheless additional administration of albumin to the batches did not explain this effect (data unpublished). Therefore a limited detoxification based on a single (or more than one) enzyme might be an explanation, especially when the toxic compound is administered in high concentrations (leading to a detoxification kinetic of zero order).

To clarify this issue investigations were done with cells in suspension so that different or the same cell numbers could be examined per each experiment and a variation of enzyme activities by changing cell numbers could be assessed. Furthermore changes in the operative surface area can be appraised comparing the present results to results of previous experiments with monolayers. If experiments would be performed with monolayers of cells a changed cell number per batch would typically change the functional status of the cells. Therefore conclusions would be very hard to be drawn.

In this work we choose 100 μmol/l HC or 7.5 μmol/l DEX as the GC concentration for the pre-treatment procedure. The parameters to assess toxicity were inhibition of MET incorporation, glutathione depletion as well as the increase of oxidized glutathione expressed as oxidized glutathione/total glutathione ratio. L2 and A549 cells were used as models, both known to exert alveolar epithelial-like characteristics.

2. Material and methods

2.1. Chemicals

Cell culture chemicals (DMEMmF12, MEM (Hanks' salts), MEM (Earle's salts), penicillin/streptomycin, MET 100×, trypsin/EDTA) were obtained from Invitrogen (Eggenstein, Germany), fcs from Biochrom (Berlin, Germany), glutamine, cysteine and Trypan Blue from Merck (Darmstadt, Germany). DTNB, trypsin inhibitor, and GSSG were purchased from Sigma (Deisenhofen, Germany), NADPH, and GSSG reductase (EC 1.6.4.2) from Roche Diagnostics (Mannheim, Germany), 2-vinylpyridine from Aldrich (Steinheim, Germany), ^{35}S-MET/CYS mixture from Hartmann Analytic (Braunschweig, Germany), and Coomassie blue from Serva (Heidelberg, Germany). All other reagents were from Merck (Darmstadt, Germany).

2.2. Cell culture

The cell lines (A549, L2; alveolar epithelial type II-like) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were grown in DMEMmF-12 (supplemented with 50 IE/ml penicillin, 50 μg/ml streptomycin and 10% fcs (L2) or 5% fcs (A549) in a moist CO_2 (5% v/v) atmosphere at 37 °C. The cells were passaged weekly using a standard trypsin/EDTA protocol (Freshney, 1987). Medium was changed every other day and 14 h before zinc exposure.

2.3. Exposure protocol and methionine incorporation assay

Investigations with confluent monolayers of cells were done in 24 well plates with about 50–100 μg cellular protein per each batch. Investigations with cells in suspensions were performed in 2.0 ml reaction tubes, containing 1.0 ml cell suspensions. Viability of cells was 85 ± 14% as assessed by Trypan Blue dye exclusion.

2.4. Pretreatment procedure

As indicated alveolar epithelial cells were pretreated with 100 μmol/l HC (0.1% ethanol), or 7.5 μmol/l DEX (0.03% ethanol) for 72 h (controls corresponding 0.1% or 0.03% ethanol, resp.), or with up to 100 μmol/l BSO for 14 h in culture medium with fcs.

2.5. Exposure procedure

For experiments with cells in suspensions cell layers were washed once with HBSS (without Ca_2^+ or Mg^{2+}) then detached from the surface with trypsin. Trypsin activity was stopped with trypsin inhibitor and cells were washed with MEM (Earle's salts). Cells were resuspended and then incubated for 3 h with six or seven concentrations of zinc chloride (0, 20–500 μmol/l), tBHP (0, 2–200 μmol/l) or HP (L2 cells: 0, 7.5–500 μmol/l (2 h); A549 cells: 0, 50–650 μmol/l) in MEM (Earle's salts, MET: 5 μmol/l, CYS: 10 μmol/l) at 37 °C in 5% (v/v) CO_2 atmosphere with continual rotation (<1 g) to avoid sedimentation of cells. The concentrations were chosen to get at least two concentrations higher or lower compared to the estimated EC_{50} value. Then radiolabeled MET (MET = MET/CYS-mixture, 75/25%) (^{35}S: 2 μCi per ml) was added in MEM (Earle's salts, MET and CYS as indicated above) for 1 h at the end of the incubation period. Incubation was terminated by centrifugation (200 g) and medium was discarded. Cell pellets were rinsed with ice-cold MEM to remove excess label and centrifuged a second time.

Incubation of cells for monolayer experiments with zinc chloride, tBHP or HP was performed in MEM (Earle's salts, MET: 5 μmol/l, CYS: 10 μmol/l) at 37 °C in 5% (v/v) CO_2 atmosphere as indicated after the cell layers had been washed with HBSS (including Ca_2^+ and Mg^{2+}). As in the investigations with cells in suspension seven concentrations of zinc chloride (0, 15–200 μmol/l) for 2 h, or tBHP (L2 cells: 0, 50–300 μmol/l; A549 cells: 0, 50–750 μmol/l) for 3 h, or HP (L2 cells: 0, 150–2000 μmol/l; A549 cells: 0, 100–8000 μmol/l) for 3 h were used and the corresponding EC_{50} value was estimated for each group. In monolayer experiments incubation was terminated by discarding the medium and washing the cells with ice-cold MEM.

Afterwards the cells were treated with ice-cold 0.33 mol/l $HClO_4$. The supernatant was assessed for glutathione content and acid-soluble radioactivity. The precipitate was dissolved in NaOH (0.5 mol/l incl. 1% sodium laurylsulfate (SDS)) and acid-insoluble radioactivity was measured. One batch of cells per each group was used to determine the glutathione content. These cells were lysed without incubation with the pro-oxidant substances.

2.6. Measurement of glutathione content

Total cellular glutathione and oxidized glutathione (GSSG) were measured in neutralized cell extracts using the Tietze assay with DTNB, GR, and NADPH (Tietze, 1969). The analytical detection limit for glutathione was ≈0.2 nmol per mg of cellular protein (Walther, 2004). GSSG was determined after GSH had reacted with 2-vinylpyridine (2 μl per 100 μl of acid cell extract at pH 7) for half an hour. Then excess vinylpyridine was removed by evaporation. Calibration was performed with freshly prepared acidic GSSG

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