Interspecies Comparison of Rat and Hamster Alveolar Macrophage Antioxidative and Oxidative Capacity

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Generation of oxidants has been implicated in lung injury and disease caused by a variety of inhaled agents such as ozone, particles, and mineral fibers. Antioxidants in the pulmonary system presumably provide the initial defense against such oxidants. We designed the present study to assess the oxidative and antioxidative capacity of alveolar macrophages (AM) from rats and hamsters. These two laboratory animal species commonly used in biomedical research are well known for their disparate response to pulmonary irritants/toxicants. AM from CD rats and Syrian golden hamsters were obtained by bronchoalveolar lavage. We assessed AM antioxidative levels by measuring the catalase and superoxide dismutase (SOD) activity and the intracellular concentrations of total glutathione, ascorbic acid, and α-tocopherol. We determined the AM oxidative capacity by assessing the ability of AM to oxidize extracellular glutathione (GSH) and to release superoxide anions. There were no significant differences in the intracellular antioxidative levels, except for catalase activity that was significantly (p<0.05) higher in hamster AM than in rat AM. However, AM oxidative capacity was markedly different between the two species studied. The amount of spontaneous and phorbol myristate acetate (PMA)-induced GSH oxidation was about 5-fold higher in rat AM than in hamster AM, whereas the PMA-induced superoxide anion release did not differ significantly between the two rodents. In summary, our data suggest that species variation exists between the oxidative capacity of rat and that of hamster AM. Whereas the oxidative capacity of hamster AM appears to be based mainly on the formation of reactive oxygen species, it is suggested that rat AM possess an additional oxidative system.

Methods

Animals

Male CD rats (Crl:CD(SN)BR; 250–300 g) and male Syrian golden hamsters (Lak: LVG(STR)BR; 120–150 g) were obtained from Charles River (Sulzfeld, Germany) and kept in a conventional, nonbarrier rodent housing unit. Water and standard rodent laboratory diets supplemented with 18,000 IU/kg vitamin A, 1280 IU/kg vitamin D3, and 120 mg/kg vitamin E (smiffl, Soest, Germany) were supplied ad libitum.

Cell Isolation

AM were obtained by bronchoalveolar lavage as described previously (9). The animals were anesthetized by ip injection of sodium pentobarbital (rat, 30 mg/kg bw; hamster, 24 mg/kg bw). After cannulation of the trachea, the thorax was opened, and the lungs were mobilized. The lungs of hamsters were lavaged with ten 5-ml aliquots and the lungs of rats with ten 10-ml aliquots of sterile, nonpyrogenic phosphate-buffered saline solution (PBS) (Serva, Heidelberg, Germany). After centrifugation of the pooled samples at 300×g for 10 min, the cell pellet was washed twice and resuspended in RPMI 1640 (Seromed, Munich, Germany) supplemented with

Introduction

Oxidants such as reactive oxygen species and other free radicals may be important causes of lung injury and diseases including adult respiratory distress syndrome, pulmonary fibrosis, chronic bronchitis, and emphysema (J). Oxygen species, formed during the reduction of molecular oxygen, can be generated by a diverse group of exogenous inhaled agents such as ozone, particles, or mineral fibers (2,3). Effects of reactive oxygen species are counterbalanced by an elaborate system of enzymatic and nonenzymatic antioxidants (4). In the removal of reactive oxygen species and other radicals, antioxidants such as superoxide dismutase (SOD), catalase, glutathione (GSH), ascorbic acid, and α-tocopherol are involved. Under normal circumstances, these antioxidants protect cells and tissue from oxidative damage, but with a large or long oxidative challenge, these defenses will be depleted and injurious responses may ensue (5).

We designed the present study to investigate the oxidative capacity and antioxidant level of alveolar macrophages (AM) from rats and hamsters. AM constitute one of the first lines of cellular defense against inhaled pathogenic material and possess a high phagocytic and microbicidal potential. Rats and hamsters are two laboratory animal species commonly used in biomedical research and are well known for their disparate response to pulmonary irritants/toxicants such as pure oxygen (6), diesel soot (7), or mineral fibers (8). To assess the antioxidant level of AM, we measured the intracellular concentrations of total GSH, ascorbic acid, and α-tocopherol and the SOD and catalase activity. We determined AM oxidative capacity by assessing the ability of AM to oxidize extracellular GSH and to release superoxide anion upon stimulation with phorbol myristate acetate (PMA).

Key words: macrophages, alveolar macrophages, glutathione, superoxide anion, superoxide dismutase, catalase, rat, hamster, species differences

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Abbreviations used: AM, alveolar macrophages; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; GSH, glutathione; PMA, phorbol myristate acetate; SOD, superoxide dismutase; GSH, total glutathione.
l-glutamine and gentamycin (0.16 mg/ml). Total cell counts were assessed with a standard hemacytometer (Coulter Electronics, Krefeld, Germany). After staining with May–Grunwald–Giemsa, cell populations were identified using air-dried cyt centrifuge smears (500 rpm × 5 min). The preparations contained approximately 90 to 100% AM, as characterized by morphologic criteria. The cell viability was determined by trypan blue exclusion and was greater than 90% viable AM.

**Measurement of Superoxide Anion Release**

Superoxide anion release was measured by the SOD-inhibitable reduction of ferricytochrome c as previously described (10). The cells (0.2 × 10⁶/well) were plated to 96-well flat-bottomed cell culture plates (Nunclon Delta, Roskilde, Denmark) and cultured to adhere for 2 hr at 37°C and 5% CO₂/95% air. The nonadherent cells were removed and the adherent AM were incubated at 37°C in Hank’s balanced salt solution containing 160 μM cytochrome c (Sigma Chemie, Taufkirchen, Germany) in the absence or presence of 300 U/ml SOD (Sigma) and 75 nM PMA (Sigma). Absorbance was determined after 60 min of incubation time spectrophotometrically at 550 nm (Ear 400 AT, SLT Labinstruments, Salzburg, Austria). The amount of superoxide anion formed was calculated using an extinction coefficient of 21 × 10⁻³ M⁻¹ cm⁻¹ (10).

**Measurement of Total Glutathione**

Total glutathione (GSH₃) was measured as described previously (11). The cells (1 × 10⁶ /well) were plated to 6-well flat-bottomed plastic culture dishes and cultured to adhere for 30 min at 37°C and 5% CO₂/95% air. After removal of nonadherent cells, AM were lysed and mixed with 1.1 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM ethylenediamine tetraacetic acid (EDTA), 0.2 mM NADPH, 63.5 μM 5, 5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Serva, Heidelberg, Germany), and 4 U/ml glutathione reductase (Sigma). The rate of reduction of DTNB was recorded at a wavelength of 412 nm (Lambda 19 Spectrometer, Perkin Elmer, Überlingen, Germany). The GSH concentration was calculated using an internal standard of 0.84 μM GSH (Sigma).

**Measurement of GSH Oxidation**

The GSH oxidation assay was performed as described previously (11). Briefly, AM were incubated with 50 μM GSH and with or without 75 nM PMA at 37°C and 5% CO₂/95% air. Blanks, i.e., incubation medium without cells present, were processed in the same way. After 1 hr, the supernatant was removed, and catalase (Sigma) was added to a final concentration of 80 U/ml to stop H₂O₂-dependent reactions. The samples were mixed with DTNB at a final concentration of 250 μM, and after 5 min of incubation the absorption was measured spectrophotometrically at 412 nm. The GSH concentration was calculated using a 4-point standard curve (75, 56.25, 37.5, and 18.75 μM). GSH oxidation was calculated from the concentration in the presence of AM compared to that of the cultures without cells.

**Measurement of Superoxide Dismutase Activity**

Total SOD was determined according to McCord and Fridovich (12). Cells (3 × 10⁶ to 1 × 10⁷) in 0.5 ml 50 mM K₃-phosphate buffer were disrupted by sonication (Sonicator XL, Heat Systems, Farmingdale, NY) and centrifuged at 4000 × g for 15 min at 4°C. Enzyme activity was determined at 25°C in 50 mM K₃-phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 20 μM cytochrome c, 10 μM K₃-azide and 50 μM xanthine (Sigma). Xanthine oxidase (Sigma) was added to the reaction mixture to reduce ferricytochrome c at a rate of 0.02 absorbance U/min at 550 nm. The amount of enzyme required to inhibit this reduction by 50% was defined as one SOD unit.

**Measurement of Catalase Activity**

Catalase assay was assayed according to Aebi (13). The rate of H₂O₂ reduction was measured spectrophotometrically at 240 nm (Beckman DU 7500, Beckman Instruments, Fullerton, CA) for 1 min at 25°C.

**Measurement of Ascorbic Acid and α-Tocopherol**

Intracellular concentrations of ascorbic acid and α-tocopherol were determined by using high pressure liquid chromatography according to the method of Schuep et al. (14) and Hatam and Kayden (15).

**Statistical Procedures**

Results are presented as mean ± SEM. Data were analyzed using the Mann-Whitney rank sum test. A two-tailed p-value less than 0.05 was considered significant.

**Results**

As shown in Table 1, the intracellular concentration of GSH₃, the SOD activity, and the levels of ascorbic acid and α-tocopherol did not differ significantly among the AM from the two rodent species. Intracellular catalase activity, however, was significantly (p < 0.05) higher in hamster AM than in rat AM. The PMA-induced superoxide anion release was significantly (p < 0.05) higher than the spontaneous release in both rat (3.5 ± 0.5 vs 0.9 ± 0.6 nmol/mg protein) and hamster AM (3.4 ± 0.9 vs 0.3 ± 0.3 nmol/mg protein). However, significant differences in the superoxide anion release by AM were not detected among the two species studied. In contrast, the amount of spontaneous and PMA-induced GSH oxidation was about 5-fold higher in rat AM than in hamster AM (Figure 1). Interestingly, GSH oxidation increased

![Figure 1](image-url)
only slightly upon stimulation with PMA in both rat (11.1 ± 2.8 vs 9.9 ± 2.0 nmol/10^6 AM/hr) and hamster AM (2.2 ± 0.9 vs 1.4 ± 0.4 nmol/10^6 AM/hr).

**Discussion**

Oxidants, such as reactive oxygen and nitrogen species, are implicated in the pathogenesis of lung injury and disease caused by a variety of inhaled agents, such as ozone, particles, and mineral fibers (2,3). In normal lung tissue, an elaborate balance exists between the production of oxidants and the protective activities of several intracellular and extracellular enzymatic and nonenzymatic antioxidants. A disturbance of this balance, either through an increase in oxidant stress or compromise of antioxidant resources, can initiate a series of pathophysiologic events finally resulting in pulmonary injury and dysfunction (4). In this study, we focused on the antioxidant level and oxidative capacity of AM from rats and hamsters, two laboratory animal species that are well known for their disparate response to pulmonary irritants/toxins. The reasons for differences in their responses to inhalation of pure oxygen, diesel soot, or mineral fibers (6-8) are not clearly understood; but differences in cellular defenses among species may be important in providing varying degrees of protection.

To assess the antioxidant capacity of AM, we determined the intracellular activities of the enzymes SOD and catalase, and the intracellular concentrations of GSHt, ascorbic acid, and α-tocopherol. The enzyme activities measured were resting levels of activity in normal animals, and specific stimuli to induce antioxidant enzyme activities were not applied. Our data indicate that major differences in the intracellular concentration of GSHt and the intracellular activity of SOD, two antioxidants involved in the removal of superoxide anions and peroxides, do not exist among the AM from the two species studied. Moreover, the intracellular concentrations of the antioxidant vitamins ascorbic acid (vitamin C) and α-tocopherol (vitamin E) were also comparable in rat and hamster AM. In contrast, the intracellular activity of catalase, an enzyme that detoxifies hydrogen peroxide, was significantly higher in hamster AM than in rat AM. These findings are supported by an earlier report demonstrating that hamster lungs exhibited a higher catalase activity than rat lungs, whereas SOD activity was similar for lungs from both species. It has been suggested that the species differences found in lung catalase activity play a role in the increased survival of hamsters during hyperoxic exposure compared to that of rats (6).

The oxidative capacity of AM was assessed by measuring their ability to release superoxide anions and to oxidize extracellular GSH upon stimulation with PMA. The GSH oxidation assay applied by Behr at al. (11) was developed to mimic the in vivo situation by measuring the oxidative effect using GSH as a pathophysiologically relevant substrate. Our results confirm previous findings that AM from both species released increased amounts of superoxide anions upon stimulation with PMA (16-18). However, no differences in the spontaneous and PMA-induced superoxide anion release were detected among the AM from both rodents.

In striking contrast, the levels of both spontaneous and PMA-induced oxidation of extracellular GSH were about 5 times higher in rat than in hamster AM. To estimate to what extent oxygen radicals could account for the high amount of GSH oxidized by AM, we calculated the ratio of nanomoles of superoxide anions generated and nanomoles of GSH oxidized by AM. This ratio proved to be 0.3 in rat and 1.5 in hamster AM, which strongly indicated that in rat AM the generation of reactive oxygen species was unlikely to be the only source of GSH oxidation. In previous work, we have reported that rat AM produced nitric oxide upon stimulation with lipopolysaccharide and/or interferon-γ, whereas hamster AM lacked the ability to generate nitric oxide upon the same treatment (9). Nitric oxide, as a reactive nitrogen species, interacts with GSH to yield S-nitrosoglutathione (19). Thus, interspecies differences in nitric oxide formation by AM from rats and hamsters may account for the observed discrepancy in their capacity to oxidize extracellular glutathione.

Taken together, our data suggest that major differences in the antioxidant capacity of AM from the two rodent species do not exist. However, our findings indicate marked species variation in the oxidative capacity of rat and hamster AM. Whereas the oxidative capacity of hamster AM seems to be based mainly on the formation of superoxide anion and other reactive oxygen species, rat AM appear to possess an additional oxidative system. Whether the source of this additional oxidative system is related to nitric oxide formation, must be confirmed by further investigations.

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