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# Hydrogen Peroxide Metabolism in Alveolar Macrophages after Exposure to Hypoxia and Heat

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## Summary

High temperature can change the effects of intra- and intercellular regulators and therefore modify the cellular response to hypoxia. We investigated $H_2O_2$ production by alveolar macrophages, isolated from adult male rats, which were incubated under conditions of oxygen deficiency and high temperature (experiment *in vitro*). The incubation of these cells for 2 hours at 10 % or 5 % oxygen led only to slight fluctuations in the $H_2O_2$ level, while the rise of temperature from 37°C up to 42°C significantly increased its generation. Level of thiobarbituric acid-reactive substances (TBARS) underwent similar changes. Under these conditions the accumulation of $H_2O_2$ was found to be caused mainly by its decreased cleavage rather than its enhanced production. This is indicated by decreased catalase and glutathione peroxidase activity together with a parallel absence of significant changes in superoxide dismutase (SOD) activity. Slight fluctuation of reduced glutathione level and the pronounced increase of glucose-6-phosphate dehydrogenase (G6PD) activity were detected. Strong (5 %) but not moderate (10 %) lack of oxygen led to a sharp increase in formation of cellular nitrite ions by alveolar macrophages. In general, our data showed that high temperature did not lead to any qualitative shifts of defined hypoxia-derived changes in oxidant/antioxidant balance in alveolar macrophages, but promoted sensitivity of cells to oxygen shortage.

**Key words**

Alveolar macrophages • Hypoxia • Hyperthermia • Hydrogen peroxide • Free radicals

## Introduction

Hypoxia may be an important cause and a consequence of lung injury because it is accompanied by harmful action of reactive oxygen species (ROS) and triggers lipid peroxidation in biological membranes (Tuohy *et al*. 1993, Wilhelm *et al*. 1999). Among diverse oxidants underlying this phenomenon hydrogen peroxide ($H_2O_2$) has a key significance (Kinnula *et al*. 1995, Wilhelm *et al*. 1999).

$H_2O_2$ takes part in many biochemical and physiological processes but in excessive amount it exerts harmful effects. Its ability to cross cell membranes by simple diffusion allows promoting radical reaction on great distance from its origin. Cooperating with transition metal ions it converts to hydroxyl radicals, which have been considered to be the most reactive and potentially dangerous (Cheknev 1999).

The $H_2O_2$ pool in cells is mainly derived from the dismutation of superoxide anion, which in turn is formed in reaction of molecular oxygen reduction catalyzed by NADPH oxidase and xanthine oxidase. Another way of superoxide formation is its generation in the mitochondrial respiratory chain. Further restoration of oxygen directly or indirectly results in the formation of $H_2O_2$. This process is catalyzed by superoxide dismutase (SOD). It can also proceed spontaneously but the rate of such reaction is very slow. Formed $H_2O_2$ can be scavenged by catalase (CAT) or glutathione peroxidase (GP) to water and oxygen. It is considered that $H_2O_2$ destruction by these enzymes has inhibitory effect on hydroxyl radical formation. This phenomenon can be very important for cell survival in conditions of oxidative stress (Quinlan *et al*. 1994).

Alveolar macrophages (AM) represent the predominant lung cell type, contributing to $H_2O_2$ production. AM in hypoxic conditions are known to produce more $H_2O_2$ than AM of control animals (Wilhelm *et al*. 1996), but the mechanism of this phenomenon is still not clear. Therefore, the aim of our study was to investigate the metabolism of $H_2O_2$ in a primary culture of AM, exposed to a lack of oxygen. With the assumption that hypoxia in lung injury, especially in inflammatory diseases, is often accompanied by fever (Simon 2004), AM incubation was carried out at a temperature from 37 °C up to 42 °C.

## Methods

Alveolar macrophages were collected by bronchoalveolar lavage of lungs isolated from Wistar male rats weighing 140-180 g. The lungs were lavaged with a solution containing 140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 мМ glucose, 0.2 mM EGTA, pH 7.40. The lavage fluid was filtered and centrifuged within 10 min (900 rpm at a temperature 4 °C). Cell pellet was resuspended in nutrient DME medium (Sigma, USA) supplemented with antibiotics (penicillin, gentamycin) and L-glutamine, to make the concentration $10^6$ cells/ml.

AM were then incubated in plastic Petri dishes (Falcon, Germany) for 45 min at 37 °C. After the incubation nonadherent cells were removed and adherent AM were cultured under normoxic (5 % $CO_2$, 95 % air) or hypoxic conditions (10 % $O_2$, 5 % $CO_2$, 85 % $N_2$ or 5 % $O_2$, 5 % $CO_2$, 90 % $N_2$) and temperature range 37-42 °C. The cells were used for experiments after 2 h of incubation.

By the end of incubation period, the supernatant was gently decanted from cell culture and filtered to remove detached cells. The cell layer was harvested with scraper (Costar, USA), AM were homogenized in isotonic 0.9 % NaCl solution. Production of $H_2O_2$ by AM was determined spectrophotometrically using the reaction with tetramethylbenzidine in the presence of a horseradish peroxidase (De Benedetto *et al*. 2000). Total cellular SOD activity was measured by a method based on the oxidation of quercetin in an alkaline medium containing tetramethylethylendiamine (Kostyuk and Potapovitch 1989), GP activity was analyzed by measuring the oxidation of reduced glutathione in the presence of *t*-butylhydroperoxide (Moin 1986), glucose-6-phosphate dehydrogenase (G6PD) spectrophotometrically as described previously (Prochorova 1982). Reduced glutathione level (GSH) was estimated by Ellman reaction (Seldak and Lyndsay 1968). CAT activity was assayed according to the rate of $H_2O_2$ reduction, which was measured spectrophotometrically at 410 nm for 10 min at 25 °C (Mamontova and Beloborodova 1994). Lipid peroxides were determined as thiobarbituric acid-reactive substances (TBARS) by the method of Latinova and Goncharenko (1985). The amount of NO produced by AM was assessed by measuring of stable oxidized products of NO – nitrite ions using the Griess reaction (Green *et al*. 1982).

All the data are based on at least three independent experiments, the numerical data are presented as mean ± S.E.M. and analyzed using Student *t*-test. $P<0.05$ value was considered significant.

## Results

There was just a tendency to increase of the $H_2O_2$ concentration under the influence of $O_2$ deficiency at 37 ºC. In parallel with the rise of the cell incubation temperature (37-42 °C) $H_2O_2$ level in AM steadily increased (Fig.1). This tendency was noted both at a normoxia and after $O_2$ decrease down to 10 % or 5 %. The most prominent changes were observed at the temperature range 38-40 °C.

Under these conditions high level of $H_2O_2$ can be caused by the augmented formation of ROS by AM. It is indirectly confirmed by the observed rise of TBARS, which was more pronounced at a high temperature of AM incubation (Table 1).

**Table 1.** Effect of hypoxia and hyperthermia on TBARS, cellular and extracellular nitrite level in rat alveolar macrophages

| | TBARS, nmol/$10^6$ cells (n=6) | | | cellular $NO_2^-$, nmol/$10^6$ cells (n=6) | | | extracellular $NO_2^-$, μM (n=3) | | |
|---|---|---|---|---|---|---|---|---|---|
| °C | 21 % $O_2$ | 10 % $O_2$ | 5 % $O_2$ | 21 % $O_2$ | 10 % $O_2$ | 5% $O_2$ | 21 % $O_2$ | 10 % $O_2$ | 5 % $O_2$ |
| *37* | 1.91±0.346 | 1.82±0.191 | 1.99±0.130 | 7.5±0.21 | 7.8±0.27 | 17.3±1.57* | 11.3±1.08 | 15.0±2.55 | 23.0±1.41* |
| *38* | 2.11±0.288 | 2.42±0.483 | 2.79±0.423 | 7.6±0.78 | 7.6±0.49 | 17.5±0.69* | 11.3±0.41 | 16.0±3.67 | 20.7±2.48 |
| *39* | 3.12±0.441 | 3.16±0.697 | 3.65±0.701 | 8.3±0.37 | 8.9±0.58 | 17.9±1.03* | 12.7±0.41 | 11.7±2.68 | 20.3±1.08* |
| *40* | 3.26±0.379** | 3.34±0.690 | 3.57±0.532** | 7.5±0.76 | 8.1±1.00 | 17.0±1.77* | 13.3±0.41 | 15.3±0.41 | 26.7±0.41* |
| *41* | 3.34±0.300** | 3.43±0.669 | 3.71±0.453** | 7.8±0.46 | 9.0±1.24 | 16.9±0.55* | 12.0±1.87 | 16.0±0.71 | 20.7±4.81 |
| *42* | 3.49±0.725 | 3.63±0.474** | 4.26±0.541** | 7.9±1.99 | 11.9±1.45** | 18.0±1.32* | 11.7±0.82 | 16.7±0.82 | 23±0* |

* - P<0.05 vs. mean at 21 % O2 and the same temperature, ** - P<0.05 vs. mean at 37 ºC and the same $O_2$ concentration in surrounding space.

Neither low $O_2$ concentration in the environment nor overheating to 42 °C triggered changes in the activity of SOD, a key enzyme of $H_2O_2$ formation (Fig. 1). Simultaneously with the changes of $H_2O_2$ an increase of G6PD activity was observed. A reduction of $O_2$ levels in the surrounding medium resulted in the steadily increase of G6PD activity even at 37 °C. At 5 % $O_2$ the activity of G6PD was more than 70 % higher than at 21 % $O_2$. Further rise of temperature enhanced the sensitivity of the enzyme to a lack of oxygen. The augmentation of its activity thus exceeded 100 % (Fig. 2).

NADPH formed in G6PD reactions can be used not only for reactive oxygen species (ROS) formation in reaction catalyzed by NADPH oxidase, but also for formation GSH, which serves as an important component of GP reaction. GP catalyzes the reaction of $H_2O_2$ cleavage to water and molecular oxygen. Activity of this enzyme in AM cultivated at 37 °C was attenuated with the decrease of oxygen concentration in the environment. Similar changes were observed under $O_2$ deficiency at higher incubation temperature (38-42 °C). However, it did not mean that the temperature itself had no influence on GP activity. With the rise of temperature GP activity also slightly decreased. Thus the activity of GP at 5 % $O_2$ and 37 °C was approximately identical to its value at 10 % $O_2$ and 42 °C and at 5 % $O_2$ and 42 °C it amounted only to 45 % of that seen at 21 % $O_2$ and 37 °C (Fig. 1).

The increased formation of NADPH should promote the activity of glutathione reductase (GR) and therefore should increase GSH levels, because NADPH is used as a coenzyme in the reaction reducing glutathione. Moreover, the observed decrease of GP activity under these conditions suggested a reduced consumption of GSH for $H_2O_2$ destruction. However, GSH level was not increased. In contrast, it was even decreased at 38 °C and low $O_2$ concentration (Fig. 2).

During the experiments we also detected sharp changes of nitrites produced by AM. At 5 % $O_2$ it was practically doubled in comparison to cells incubated at normal $O_2$ level, whereas there was only a tendency to an increase at 10 % $O_2$. This dependence was maintained during the rise of incubation temperature and included both cellular and extracellular level of nitriteions. The incubation temperature alone did not influence nitrite concentration generated by AM (Table 1).

## Discussion

Recently, it was demonstrated that hypoxia is accompanied by increased ROS production, which mediates lipid peroxidation in membrane structures (Vanden Hoek *et al*. 1998, Wilhelm and Herget 1999, Paddenberg *et al*. 2003). Hydrogen peroxide is supposed to play the key role in this process (Kinnula *et al*. 1995). On the basis of the hypothesis that hyperthermia is capable of causing cellular hypoxia and triggers metabolic stress with formation of ROS in the organism (Hall *et al*. 1999), high temperature was suggested to promote hypoxia-induced accumulation of $H_2O_2$.

AM are the major source of $H_2O_2$ in the lungs, because ROS formation in these cells is necessary for their phagocytic function (Wilhelm *et al*. 2003). Our results demonstrated that a rise of temperature in the surrounding space could really promote the increase of $H_2O_2$ accumulation by AM. The sensitivity of cells to the increased temperature was comparable with their sensitivity to the lowered $O_2$ concentration in the environment. The effect was intensified when AM were

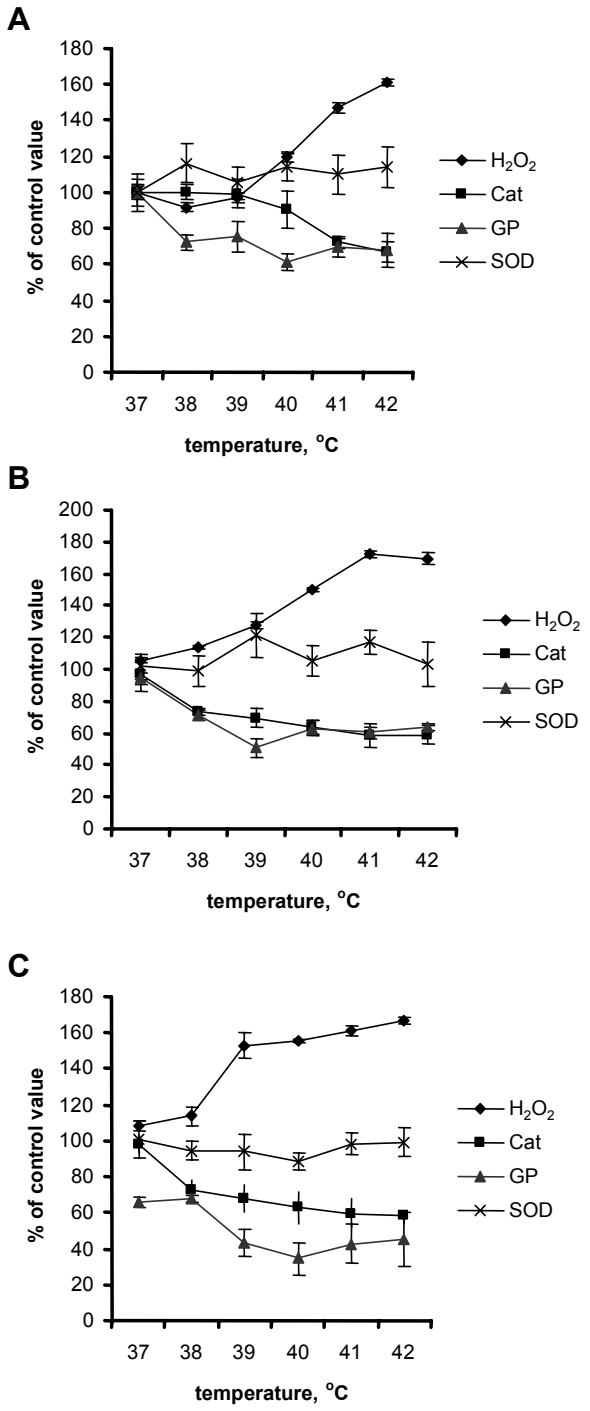


**Fig. 1.** Hydrogen peroxide level and activity of its metabolic enzymes in rat alveolar macrophages after cell exposure to hypoxia and high temperature. All parameters were measured spectrophotometrically from intact alveolar macrophages, which were incubated 2 h at different temperature and 21 % (**A**), 10 % (**B**), 5 % (**C**) $O_2$ in surrounding space. Data are the result of a representative experiment that was repeated 4 to 6 times. 100 % represent enzyme activity or $H_2O_2$ level in control experiments (21 % $O_2$, 37 °C). All data represented as means ± S.E.M.

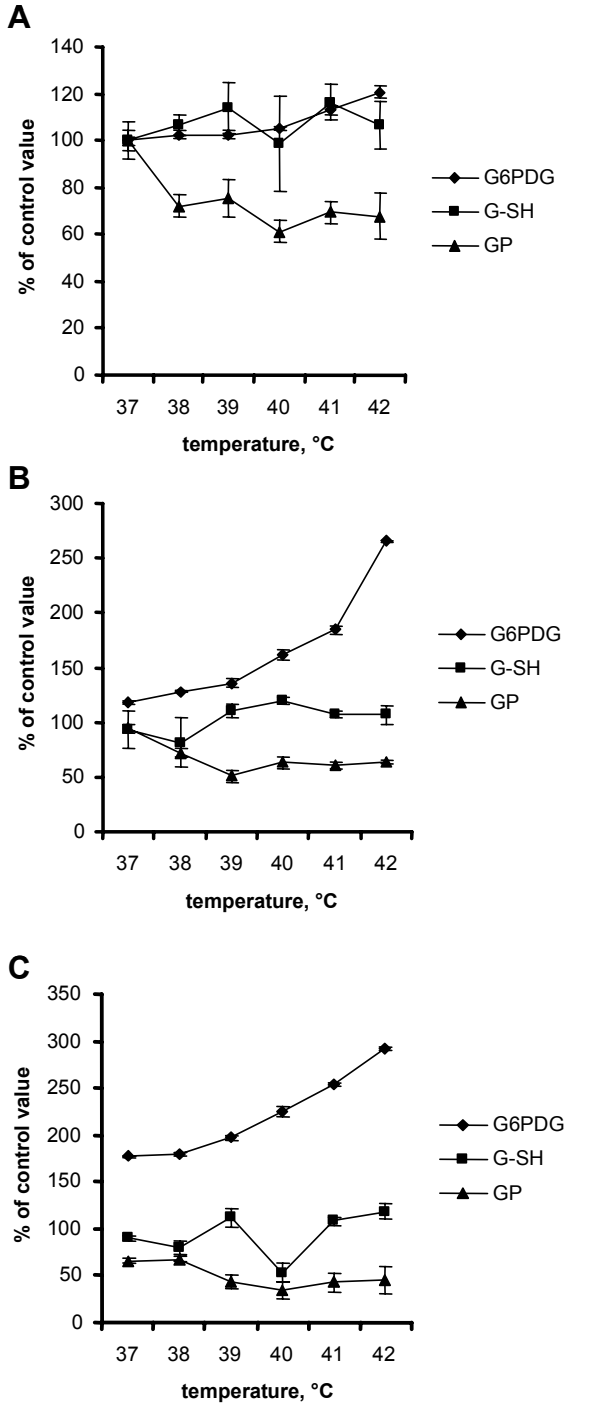


**Fig. 2.** G6PD, GP activity and GSH level in rat alveolar macrophages incubated at a shortage of $O_2$ and high temperature. All parameters were measured spectrophotometrically from intact alveolar macrophages, which were incubated for 2 h at different temperature and 21 % (**A**), 10 % (**B**), 5 % (**C**) $O_2$ in surrounding space. Data are the result of a representative experiment that was repeated 6 times. 100 % represent enzyme activity or GSH level in control experiments (21 % $O_2$, 37 °C). All data represented as means ± S.E.M.

exposed to both oxygen lack and raised temperature. This might explain the highest $H_2O_2$ levels at temperature 41-42 °C and 5-10 % $O_2$.

The source of the increased $H_2O_2$ formation could be the augmented quantity of superoxide anion radical as well as the increased NADPH oxidase or SOD

activity. Indeed, hypoxia is capable to increase the superoxide release in mitochondria mainly due to the second complex of respiratory chain because at low partial pressure of oxygen the catalytic activity of succinate dehydrogenase switches to fumarate dehydrogenase (Vanden Hoek *et al*. 1998, Paddenberg *et al*. 2003). On the other hand, NADPH oxidase activation has been shown in smooth muscle cells of pulmonary arteries during hypoxic exposure (Marshall *et al*. 1996).

The observed growth of G6PD activity, the key enzyme of hexose monophosphate pathway in AM could lead to an increased NADPH supply. NADPH is used in different reactions including the functioning of NADPH oxidase. Besides, intensive formation of TBARS is found to be in AM during their incubation at $O_2$ deficiency and overheating. This suggests the activation of oxidation processes, particularly lipid peroxidation, as a result of increased ROS generation.

SOD activity in our experiments did not vary in AM either under the influence of $O_2$ deficit, or due to the influence of high temperature. These data disprove the hypothesis of participation of SOD activity in the increased formation of $H_2O_2$ by AM.

The most probable cause of $H_2O_2$ increase in AM at hypoxia and/or hyperthermia is the change of its cleavage. Decreased CAT and GP activity was found to occur. The low activity of the latter enzyme is supposed to lead to less intensive expenditure of reduced glutathione. Indeed, the rise of temperature of cell incubation was found to be accompanied by a marked tendency to increased GSH levels. On the contrary, lack of oxygen led to a decrease of GSH concentration, especially at 37-38 °C. However, this does not match with registered rise of NADPH level under the abpve mentioned conditions, which would be required for maintenance of glutathione reduction catalyzed by GR.

The increased NADPH level could be responsible, at least partially, for the augmented NO production by AM at $O_2$ deficiency (Lobanova *et al*. 2005). NADPH was shown to stimulate the inducible NO synthase in neuroglia cells (Won *et al*. 2003). On the other hand, higher NO production by AM can be one of the possible reasons of changed GSH concentration in these cells due to a metabolic interrelationship between cellular GSH and NO. The rate of the reaction between superoxide anion and NO results in the formation of peroxynitrite, which exceeds superoxide anion dismutation (Beckman and Koppenol 1996). Peroxynitrite is a strong oxidizer and can directly oxidize sulfhydric groups of disulfides. This reaction leads to inhibition of various enzymes and to a decrease of GSH level (Veselá and Wilhelm 2002).

Both intrinsic and secreted nitrite ions are augmented mainly during a shortage of $O_2$. Therefore the diminished GSH level in AM under these conditions can be caused by an increased amount of NO. This is indirectly supported by the fact that heat did not lead to any changes of nitrites. Under the same conditions GSH level was not decreased and there was even a tendency to its rise. Other researchers did not also reveal any influence of hyperthermia on NO production by AM (Heidemann *et al*. 2000).

The present data showed that overheating amplifies shifts of defined parameters in AM, which were estimated for a low $O_2$ concentration. Manthous *et al*. (1995) reported that a rise of temperature by 1 °C augments oxygen consumption by 10 %. This might explain the enhanced sensitivity of overheated AM to a shortage of oxygen.

Taken together, the presented data indicate that the increase of hydrogen peroxide in alveolar macrophages at $O_2$ deficiency and especially in combination with high temperature of incubation is caused mainly by decreased $H_2O_2$ cleavage. The accumulation of peroxides in cells takes place simultaneously with the activation of both hexose monophosphate pathway of glucose oxidation and NO formation. Apparently control mechanisms responsible for NO and GSH metabolism in AM under hypoxia and hyperthermia are different. They are most sensitive to even a short-term lack of oxygen.

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