Pulmonary Xanthine Oxidase Activity of Rats Exposed to Prolonged Immobilization Stress

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Abstract. This study was designed to study xanthine oxidase (XO) and xanthine dehydrogenase (XD) activity in the lung of rats expozed to prolonged restraining immobilization stress. Immobilization caused more than twofold increase of xanthine oxidase activity in the rat lung. The activity of xanthine oxidase decreased in lung homogenates incubated at −20 °C for 24 h. The same incubation of homogenates from control rats caused a non significant increase of the activity. No measurable NAD^+ dependent xanthine dehydrogenase activity could be established in the lungs of both control rats and rats subjected to immobilization. All rats revealed methylene blue-dependent xanthine dehydrogenase activity which was more than two times higher in the immobilized animals. Incubation at −20 °C for 24 h increased the methylene blue dependent xanthine dehydrogenase activity in homogenates from control rats and decreased the enzyme activity in homogenates from immobilized rats. A working hypothesis was proposed for the sequence of events explaining the results obtained: XO – catalyzed generation of activated oxygen species may take place in the initiation of lipid peroxidation in the lung of rats immobilized for prolonged periods of time.

Key words: Immobilization stress — Lung — Xanthine oxidase — Xanthine dehydrogenase — Activated oxygen

Introduction

Our previous works (Kovacheva et al. 1994, Kovacheva and Ribarov 1995) have demonstrated a well expressed lipid peroxidation in the lungs of rats exposed to immobilization stress. The mechanism(s) of this phenomenon is not clear. Several factors may contribute to the triggering of lipid peroxidation including stress-induced high levels of catecholamines (Kvetnansky and Mikulaj 1970, Selye 1976

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Haggendal et al 1987), release of transition metal complexes from different storage sites (Della Corte and Stirpe 1972, Engerson et al 1987), metabolic acidosis (Pojarov et al 1990), ischemia, etc

Xanthine oxidase (xanthine-oxygen oxidoreductase EC 1 2 3 2) is at the end of the catabolic sequence of purine nucleotide metabolism in humans and a few other uricotelic species (Kizaki and Sakurada 1989) Although the main physiological function of xanthine oxidase remains unclear there is growing interest in the ability of this enzyme to serve as a source of oxidizing agents such as hydrogen peroxide and superoxide radicals The interest in these functions of the enzyme (as a source of oxidizing agents) has increased markedly since it has been implicated in the pathogenesis of ischemia-reperfusion injury of tissues such as heart liver kidney intestine, skin skeletal muscle, and brain (McCord 1985 Beckman 1986 Engerson et al 1987 Beckman et al 1989)

In vivo under normal conditions, the xanthine utilizing enzyme exists predominantly as xanthine dehydrogenase It utilizes NAD^+ or methylene blue rather than oxygen as an electron acceptor (Stirpe and Della Corte 1969 Della Corte et al 1969 Della Corte and Stirpe 1972, Clare et al 1981, McKelvey et al 1988) In certain conditions such as ischemia (Stirpe and Della Corte 1969, Della Corte et al 1969 Della Corte and Stirpe 1972, Clare et al 1981, Parks and Granger 1986 McKelvey et al 1988) acute ethanol administration (Sultatos 1988 Kato et al 1990) etc however the dehydrogenase form of the enzyme may be converted to oxidase form In the presence of O_2 it generates superoxide radicals and hydrogen peroxide (Stirpe and Della Corte 1969, Della Corte and Stirpe 1972, McCord 1985 Parks and Granger 1986, Beckman 1986, Engerson et al 1987) This process is dangerous because the generated activated oxygen species can participate in reactions leading to initiation of lipid peroxidation and damage to the cell membrane (Slater 1984 McCord 1985 Kagan 1988, Suzuki and Sudo 1989)

The present work was carried out in an attempt to shed some light on the mechanism of initiation of lipid peroxidation in the lung of rats exposed to acute prolonged immobilization stress In particular, the immobilization-induced changes of the lung xanthine dehydrogenase activity and xanthine oxidase activity were investigated

Materials and Methods

Sixteen Wistar male rats (200 ± 20 g) were used The animals were divided into two groups (i) 10 rats were exposed to stress after 6 hours of deprivation of food and water by immobilizing each animal in a specially designed plexiglas rest-rainer for 30 h (ii) the remaining 6 rats were not immobilized and used as a control group All animals were killed by cervical extension Then lungs were quickly removed the blood was washed out the tissue was rinsed in cold saline and homogenized in 0 1

mol/l Tris-HCl buffer, pH 8.1 (1g of liver + 5 ml of buffer), in a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 800 × g for 20 min and the supernatant was centrifuged again at $100,000 \times g$ for 1 h. The clear supernatant was continuous-flow-dialyzed for 3 h against 300 vol of the same buffer. All procedures were carried out at 4 °C.

The enzyme activity was estimated as uric acid production for 30 min. The assay was carried out in a mixture containing, in a final volume of 3 ml: 0.1 mol/l Tris-HCl buffer, pH 8.1, 60 mmol/l xanthine, 0.67 mmol/l NAD^+ or 33 mmol/l methylene blue, and 0.2 ml of lung supernatant (added last) (Stirpe and Della Corte 1969). Xanthine oxidase-catalyzed production of uric acid was stopped by addition to the mixture of 0.1 ml of 100% (w/v) trichloroacetic acid. The precipitated material was removed by centrifugation 800–900 × g. The uric acid production at 25 °C was estimated by the increase of absorbance at 292 nm, using a molar extinction coefficient of $e_{292} = 1.1 \times 10^4$ l mol^{-1} cm^{-1} (Beckman et al. 1989). Xanthine was omitted from the reference cuvettes.

The NAD^+-dependent or the methylene blue-dependent xanthine dehydrogenase activity was determined by measuring the amount of uric acid formed aerobically in the presence of NAD^+ or methylene blue (XO plus XD) with the oxygen-dependent XO activity subtracted (Della Corte and Stirpe 1970). One unit of enzyme activity is defined as the amount of the enzyme required to convert 1 mmol of xanthine to uric acid per minute, and specific activity is defined as micromoles of xanthine converted to uric acid per minute per milligram protein (Parks and Granger 1986).

Protein content was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

All reagents were of finest grade, and water was glass-distilled.

For multiple group comparisons, one-way analysis of variance (ANOVA) was employed followed by Bonferroni's test for significant differences. Statistical significance was defined at the $P < 0.05$ level. The statistical procedures were performed with InStat software version 2.1, purchased from Sigma Chemical Company (St. Louis, USA). Data were expressed as means ± S.E.M.

Results

Figure 1 presents the results concerning XO activity in lung homogenates isolated from control rats and rats exposed to prolonged immobilization stress. The enzyme activity assayed immediately after the sacrification of the animals is shown in the left panel of the Figure. The right panel presents the enzyme activity assayed in homogenates incubated at −20 °C for 24h. It is evident from the left panel that the lung XO in rats subjected to immobilization is more than two times higher compared to control rats ($P < 0.001$). Similarly, an increase of XO activity was

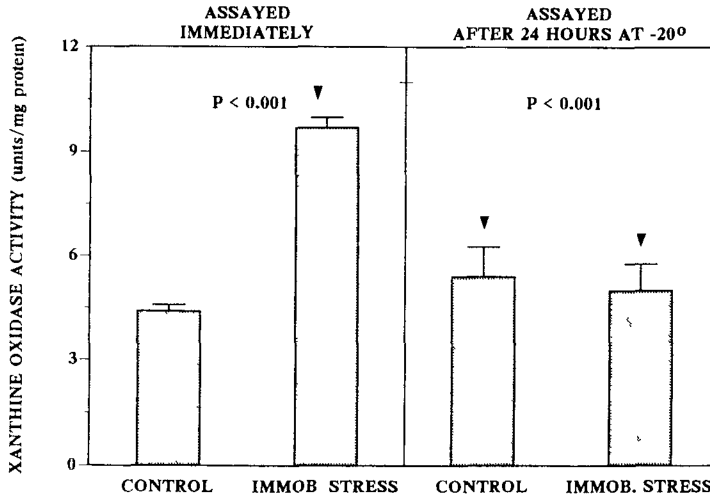

Figure 1. Xanthine oxidase activity in the lungs of control rats and rats exposed to immobilization stress. The left panel shows the enzyme activity assayed immediately after the preparation of the homogenates. The right panel shows the enzyme activity assayed in homogenates incubated at −20 °C for 24 h. The rats were immobilized for 30 h. The results are presented as mean ± S.E.M.

Table 1. Xanthine oxidase (XO) activity and NAD^+ dependent xanthine dehydrogenase (XD) activity in lung homogenates from control and immobilization-stressed rats. Effects of incubation of the homogenates at −20 °C for 24 h. The results are presented as mean ± S.E.M.

Form of the enzyme	Uric acid production (nmol/min/mg protein) Assayed immediately		Assayed after 24 h at −20 °C	
	control	immobilized	control	immobilized
XO	66.0 ± 3.0	145.5 ± 4.5	81.0 ± 13.5	75.0 ± 12.0
XO plus NAD^+ dependent XD	48.0 ± 7.5	126.0 ± 12.0	78.0 ± 7.5	72.0 ± 4.5

observed in control lung homogenates incubated at low temperature. However, the increase was significantly smaller than immobilization stress-induced increase. In contrast, the incubation at −20 °C for 24 h of lung homogenates from immobilized

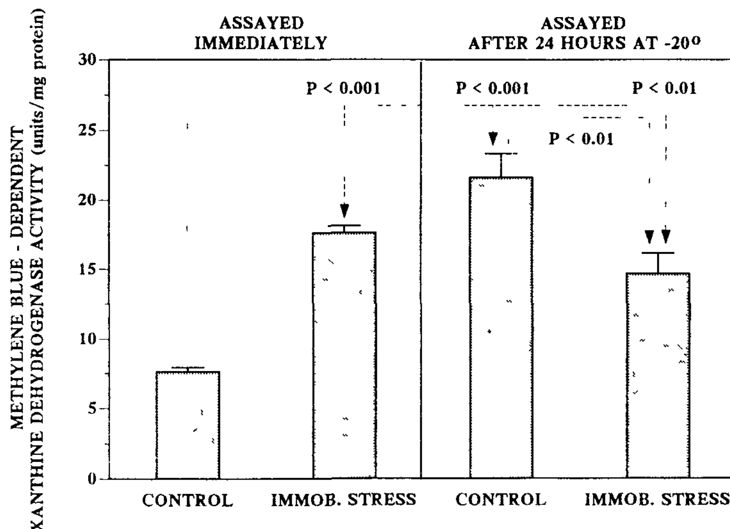

Figure 2. Methylene blue-dependent xanthine dehydrogenase activity in the lungs of control rats and rats exposed to immobilization stress. The left panel shows the enzyme activity assayed immediately after the preparation of the homogenates. The right panel shows the enzyme activity assayed in homogenates incubated at −20 °C for 24 h. The rats were immobilized for 30 h. The results are presented as mean ± S.E.M.

rats decreased significantly the XO activity to values typical of freshly prepared homogenates from control rats ($P < 0.001$).

Moreover, in presence of NAD^+ (O_2-dependent xanthine oxidase plus NAD^+-dependent xanthine dehydrogenase) the production of uric acid was slightly lower than in the absence of NAD^+ (O_2-dependent xanthine oxidase) (Table 1).

The production of uric acid in the lung homogenates from control rats increased in the presence of methylene blue. As seen in the left panel of Fig. 2, this effect was significantly stronger (more than twofold) in lung homogenates from immobilized rats. Incubation of homogenates from control rats at −20 °C greatly increased their ability for methylene blue-dependent production of uric acid. On the contrary, incubation of homogenates from immobilized rats resulted in a slight decrease of their ability of methylene blue-dependent oxidation of xanthine to uric acid.

Discussion

The results show that it is mainly XO which oxidizes xanthine to uric acid in the rat lung. The enzyme activity strongly increased as a result of acute prolonged

immobilization of the animals The mechanism of this effect is not clear It is most unlikely that there is any conversion of XD into XO because no measurable NAD^+-dependent xanthine dehydrogenase was found in the lung homogenates from both control and immobilized rats In addition if the immobilization induced increase of the lung XO were due to conversion of methylene blue-dependent XD into XO then XD would decrease On the contrary we found a significant increase of xanthine dehydrogenase as a result of immobilization of the animals

It is interesting to note that the increases (in percentages) of XO and methylene blue-dependent XD in rats subjected to immobilization was approximately the same (116% and 133% respectively) Therefore, it may be assumed as a working hypothesis that the lung xanthine-utilizing enzyme exists in a form which can use both oxygen and methylene blue as electron acceptor Similar hypothesis has been proposed earlier (Della Corte 1972 Parks and Granger 1986) for the liver xanthine utilizing enzyme Further, it seems reasonable from the biological point of view that the dehydrogenase/oxidase form of the enzyme is inhibited *in vivo* The procedures used for lung homogenate preparation possibly destroy partially the inhibitor(s) thus causing some XO and methylene blue-dependent XD activities to appear even in the controls The immobilization stress possibly destroys this inhibitor(s) to a greater extent In control homogenates this process seems to be incomplete and possibly goes on further during the incubation at low temperature On the other hand, the dehydrogenase/oxidase form of the enzyme is likely to be unstable when not inhibited Therefore, two competitive processes (i) destruction of the inhibitor and (ii) time-dependent inactivation of the produced dehydrogenase/oxidase form of the enzyme seem to take place during the incubation at low temperature In control homogenates the first process prevails It results in an increased activity of the dehydrogenase/oxidase form of the enzyme In homogenates extracted from immobilized rats (characterized by high activity of the dehydrogenase/oxidase form of the enzyme) during the incubation at low temperature the second process (enzyme inactivation) prevails It results in a decreased activity of the dehydrogenase/oxidase form of the enzyme On the other hand, it is well known that incubation of liver homogenates at -20 °C for 24h caused some conversion of XD activity into XO activity (Stirpe and Della Corte 1969, Della Corte et al 1969)

The proposed mechanism seems to be able to explain the obtained results Nevertheless it needs further experimental evidence As mentioned above, our earlier experiments (Kovacheva et al 1994 Kovacheva and Ribarov 1995) demonstrated the development of lipid peroxidation in the lungs of immobilized rats On the other hand the present work describes data showing significant increase of XO in the lungs of immobilized rats There are many data suggesting that *in vivo*, the liver xanthine-utilizing enzyme is predominantly NAD^+-dependent xanthine dehydrogenase and that it also can use methylene blue as an electron acceptor (Stirpe and Della Corte 1969, Della Corte et al 1969, Della Corte and Stirpe 1972, Clare et al

1981; McKelvey et al. 1988). In our experiments, no measureable NAD^+-dependent xanthine dehydrogenase activity could be observed.

Taking into account all the above data, it may be assumed that XO-catalyzed generation of activated oxygen species (superoxide radicals, hydrogen peroxide, singlet oxygen, etc.) may take place, at least in part, in the initiation of lipid peroxidation in the lung of immobilized rats. It is not clear, however, whether the increased XO activity is the cause for lung lipid peroxidation or the consequence of it. This problem might be resolved by investigating the immobilization-induced changes of the lung XO in rats supplemented with vitamin E. Such experiments are under way in our laboratory.

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