Errata

Immobilization Stress Enhances Lipid Peroxidation in the Rat Lungs

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Abstract. The present work was carried out to study the involvement of lipid peroxidation in immobilization-induced damage of the rat lung Thirty-hour immobilization stress was found to result in a marked morphological alteration of the lung ultrastructure and in significant increases of both acid and alkaline phosphatase for immobilization times exceeding 12 and 24 hours respectively Also, increased concentrations of conjugated dienes and fluorescent products of lipid peroxidation were measured in the lungs of rats immobilized over 12 h Immobilization stress was followed by significant changes in the fatty acid contents of lung phospholipids The levels of polyunsaturated fatty acids C-18 2 (linoleic acid) and C-20 4 (arachidonic acid) were decreased even during the alarm phase The contents of monounsaturated fatty acids did not change, while those of saturated fatty acids slightly increased The involvement of lipid peroxidation in immobilization-induced damage of the rat lung was indirectly supported by the observation of decreased levels of vitamin E at 12 h immobilization All the above data suggest that lipid peroxidation is somehow involved in the immobilization-induced damage of the rat lung The observed changes in lipid peroxidation preceded the immobilization stress-induced damage of the lung cell membranes Therefore, it seems likely that lipid peroxidation is the cause, rather than a consequence of the stress-altered lung structure

Key words: Lung — Immobilization stress — Lipid peroxidation — Vitamin E — Fatty acids — Conjugated dienes — Fluorescent products of lipid peroxidation

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~~Materials and Methods~~

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Abstract. The present work was carried out to study the involvement of lipid peroxidation in immobilization-induced damage of the rat lung. Thirty-hour immobilization stress was found to result in a marked morphological alteration of the lung ultrastructure and in significant increases of both acid and alkaline phosphatase for immobilization times exceeding 12 and 24 hours respectively. Also, increased concentrations of conjugated dienes and fluorescent products of lipid peroxidation were measured in the lungs of rats immobilized over 12 h. Immobilization stress was followed by significant changes in the fatty acid contents of lung phospholipids. The levels of polyunsaturated fatty acids C-18:2 (linoleic acid) and C-20:4 (arachidonic acid) were decreased even during the alarm phase. The contents of monounsaturated fatty acids did not change, while those of saturated fatty acids slightly increased. The involvement of lipid peroxidation in immobilization-induced damage of the rat lung was indirectly supported by the observation of decreased levels of vitamin E at 12 h immobilization. All the above data suggest that lipid peroxidation is somehow involved in the immobilization-induced damage of the rat lung. The observed changes in lipid peroxidation preceded the immobilization stress-induced damage of the lung cell membranes. Therefore, it seems likely that lipid peroxidation is the cause, rather than a consequence of the stress-altered lung structure.

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Introduction

Free radical oxidation of unsaturated lipids has become a problem of increasing interest because of its role in a variety of pathological conditions (Slater 1984; Halliwell 1987; Yagi 1987; Kagan 1988; Halliwell and Gutteridge 1989). Recently, it has been demonstrated that some forms of stress, such as exercise, starvation, trauma, major surgery, radiation, emotional and oxidation stress etc., also increase the free radical generation with subsequent initiation of lipid peroxidation (Davies et al. 1982; Meerson 1984; Demling et al. 1986; Haggendal et al.1987; Wohaieb and Godin 1987; Hidalgo et al. 1988). Furthermore, it has been proposed (Meerson 1984) that free radical processes might be implicated in the control of general physiological response to stress. This has prompted intensive studies of the involvement of free radical processes, mainly lipid peroxidation, in different stages of stress (Davies et al. 1982; Meerson 1984; Demling et al. 1986; Haggendal et al. 1987; Wohaieb and Godin 1987; Hidalgo et al. 1988; Poli et al. 1989; Sekhar et al. 1990). Large volume of data are available concerning the free radical status under stress and shock in organs such as heart, brain, liver, kidney, blood etc. (Davies et al. 1982; Meerson 1984; Haggendal et al.1987; Wohaieb and Godin 1987; Hidalgo et al. 1988; Poli et al. 1989; Sekhar et al. 1990). Lungs however seem to be less investigated in this respect (Kistler et al. 1967; Harrison et al. 1969; Adamson et al. 1970; Ratliff et al. 1970; Moss et al. 1972; Demling et al. 1986).

Immobilization stress takes up an important position among different forms of stress (Meerson 1984). Using appropriate lengths of immobilization, different stages of stress can easily be modelled in experimental studies.

The present work was carried out in an attempt to check the involvement of lipid peroxidation in immobilization stress-induced damage of the rat lung.

Materials and Methods

Animals and study design

Male white Wistar rats (200 ± 20 g) were used. The animals were maintained at standard conditions. The immobilization experiment was started after the animals had been kept without food and water for 6 hours. The rats were subjected to acute immobilization stress using the method described by Malikova and Arefalov (1982). The main stages of stress are modelled by immobilization of different length. Altogether, 198 rats were used, and they were divided into six groups. The animals of the control group (GI) were not immobilized. The immobilization times for groups GII, GIII, GIV and GV were 1, 12, 24 and 30 hours respectively. The rats of group GVI were immobilized for 30 hours with subsequent readaptation for 5 hours. According to Malikova and Arefalov (1982), the following stages of stress can be observed in experimental groups: group GII, alarm reaction; groups GIII and GIV, stage of adaptation; group GV, stage of exhaustion; group GVI, readaptation.

Electron microscopy

After the period of akinesis 3 animals from each groups were anesthetized by intramuscular injection of thiopental (20g/kg) The chest was opened the lungs were fixed and studied under electron microscope (Hitachi U-500) by the method of Low and Daniels (1952)

Assays of acid and alkaline phosphatase

The degree of the plasmalemma and lysosomal membranes integrity was estimated using the marker enzymes alkaline and acid phosphatase The methods used have been described elsewhere (Bessey et al 1946 Weissmann and Thomas 1962 Nelson 1966) In brief 10% lung tissue homogenate (w/v) in 0 25 mol/l sucrose was prepared and spun at 700 × *g* for 20 min in a refrigerated centrifuge The cell debris was kept aside and the supernatant was centrifuged again The second debris was mixed with the first one and the mixture was resuspended in sucrose The fraction containing cells and plasmatic membranes was used for analysis of alkaline phosphatase activity The supernatant was centrifuged again at 23 000 × *g* for 60 min The pellet containing the bulk of mitochondria and lysosomes was resuspended in 0 25 mol/l sucrose and was used for analysis of lysosomal acid phosphatase Activities of alkaline and acid phosphatases were assayed using *p*-nitrophenylphosphate as a substrate Both enzyme reactions were recorded spectrophotometrically using the absorbance of their product *p*-nitrophenol at 400 nm

Analysis of fluorescent products and conjugated dienes of lipid peroxidation

Lung tissue was homogenized in 0 1 mol/l K Na phosphate buffer (pH 7 4) at 4 °C Total lipids were extracted according to the method of Folch et al (1957) An aliquot of the chloroform phase was used to measure the fluorescent products of lipid peroxidation (Dillard and Tappel 1984) The light emision at 420 nm was measured using excitation light with a wavelength of 360 nm Another aliquot of the chloroform phase was evaporated The residue was dissolved in cyclohexane and used for the determination of conjugated dienes For this purpose optical absorbance at 232 nm was measured against a cyclohexane blank (Recknagel and Glende 1984) A third aliquot of the chloroform phase was used for the determination of the lipid content (Richard et al 1974)

Analysis of fatty acid composition of phospholipids

The fatty acid composition of the rat lung was analyzed by gas chromatography after conversion of the fatty acids to their respective methyl esters (Tzanev et al 1988) The measurments were performed with a Pye Unicam 104 equipment

Analysis of vitamin E

Vitamin E contents were estimated fluorimetrically by the method of Taylor et al (1976) with a slight modification The fluorescence of vitamin E after its extraction and saponification was measured at excitation and emission wavelengths of 292 nm and 325 nm, respectively All spectrophotometric and spectrofluorimetric studies were performed with a Perkin Elmer 554 Spectrophotometer and a Perkin Elmer 44B Spectrofluorimeter Quinine sulfate (1 μg/ml in 0 1 N H_2SO_4) was used as fluorescent standard The protein content was measured by the method of Lowry et al (1951) with bovine serum albumine as standard All reagents were of the finest grade and glass-distilled water was used

Statistical analysis

For multiple group comparisons, one-way analysis of variance (ANOVA) was employed followed by Bonferroni's test for honestly significant difference. Statistical significance was defined as $P < 0.05$. The statistical procedures were performed with InStat software version 2 1, purchased from Sigma Chemical Company Data were expressed as means ± S.D.M

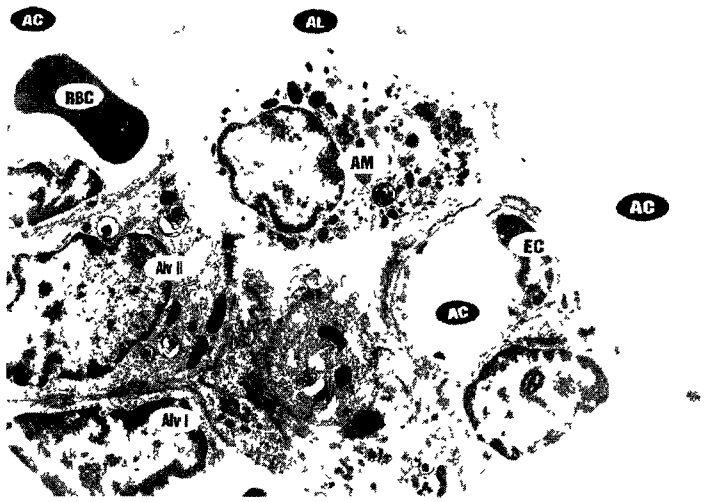

Figure 1. Section of the lung from a control animal. It shows a clean alveolar lumen (AL) and alveolar septa of normal thickness The septa contain endothelial cells (EC), alveolar type I cells (Alv I) and alveolar type II cells (Alv II) Erythrocytes (RBC) are present in the alveolar capillary (AC) There is an alveolar macrophage (AM) in the alveolar lumen (AL). (Low and Daniels' stain, ×6500).

Results

Immobilization-induced changes of the lung ultrastructure

Our initial experiments were aimed at checking the adequacy of the method selected for immobilization stress. Fig. 1 illustrates the lung ultrastructure characteristic for the control rats. The alveolo-capillary membrane has a normal structure. The alveolar walls receive adequate perfusion through numerous alveolar capillaries (AC), which consist of endothelial cells (EC) lining the basal membrane (BM) and containing erythrocytes (RBC). Thinner cytoplasmic areas of alveolar type I cells (Alv. I) and the abundant alveolar type II cells (Alv. II) can clearly be distinguished. An alveolar macrophage (AM) is present in the alveolar lumen (AL).

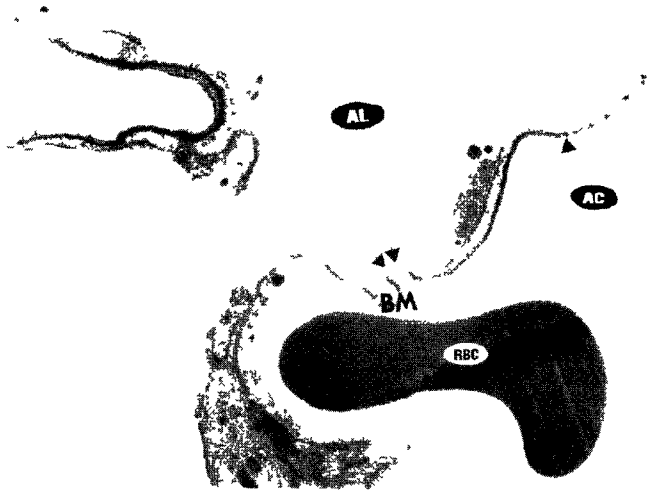

Figure 2 Section of the alveolo capillary membrane of the lung from a rat subjected to 30 hours of immobilization The membrane is damaged Endothelial cells are missing in the region marked with one arrow Some parts of the alveolo capillary membrane (two arrows) are present only as the basement membrane (BM) The cells are swelled (Low and Daniels' stain ×11 000)

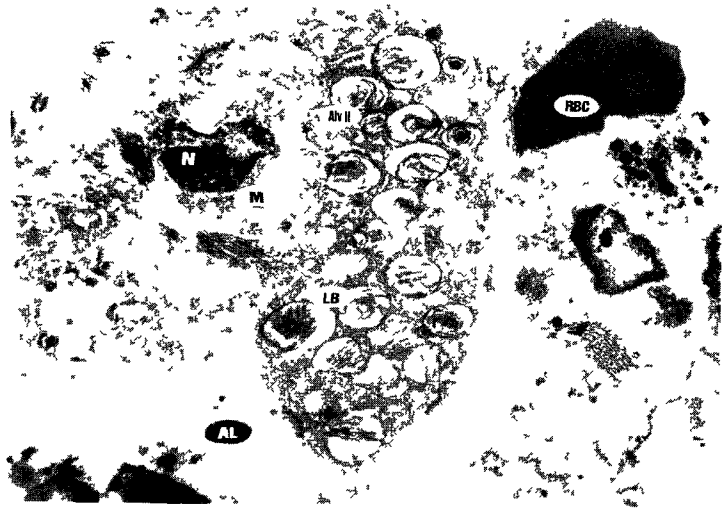

Figure 3. Rat alveolar type II cell after 30 hours of immobilization Substantial changes have occurred the cytoplasm is vacuolized, the mitochondria (M) are totally swelled and damaged, the nucleus (N) is pycnotic, a substantial part of the cytoplasm is infested with large lamellar bodies (LB) The section as a whole presents a swelled structure Microvilli are absent (Low and Daniels' stain, ×8400)

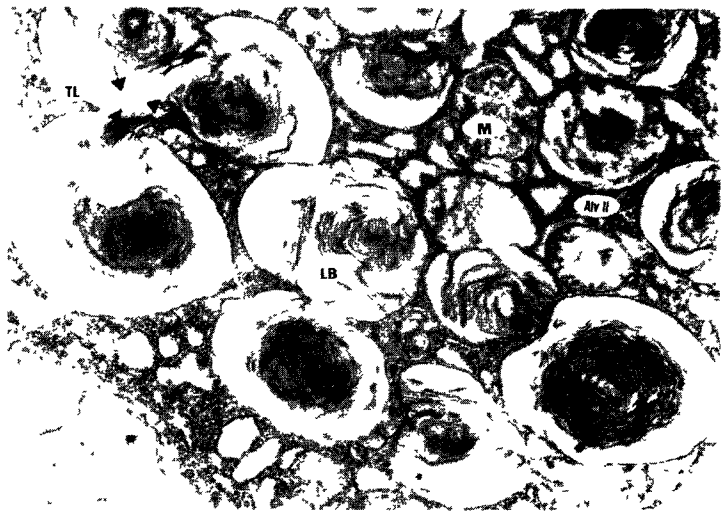

Figure 4. Section of an alveolar type II cell after 30 hours of immobilization. Some lamellar bodies (LB) reach giant size. When enclosed by the terminal lamella (TL), some lamellae are not concentric. At some points the terminal lamellae are damaged and then bodies merge (arrows). (Low and Daniels' stain, ×16,800).

Figs. 2, 3 and 4 illustrate the typical lung structure seen in rats immobilized for 30 h. In some portions of the alveolo-capillary membrane the endothelial cells are missing; the plasmalemma of some alveolar type I cells is destroyed, leaving the basement membrane stripped on both sides (Fig. 2). Alveolar type II cells are also altered (Fig. 3). Some of them have pycnotic nuclei. The development of the endoplasmatic reticulum oedema is obvious. Numerous vesicles are present due to the vacuolization of the cytoplasm. Most mitochondria are swollen; some of them show deformed shapes and destroyed cristae. The microvilli are missing. There are giant lamellar bodies. The lamellae are numerous but irregular, concentric or disordered, and separated from the terminal lamella. Occasionally, the integrity of the terminal lamella is disrupted (Fig. 4). In the cells there are aggregates consisting of 2–3 lamellar bodies. This is a pattern consistent with a well expressed damage of the alveolo-capillary membrane in the animals subjected to 30 h immobilization. The morphological changes were less manifest for shorter times of immobilization, and obviously are not suitable for studing the dependence of lung cell alterations on immobilization period. Therefore, changes of the marker enzymes alkaline and acid phosphatase, were used for this purpose.

Alkaline and acid phosphatase in the lung of rats subjected to immobilization stress

Immobilization stress- induced changes of the lysosomal-marker enzyme, acid phos-

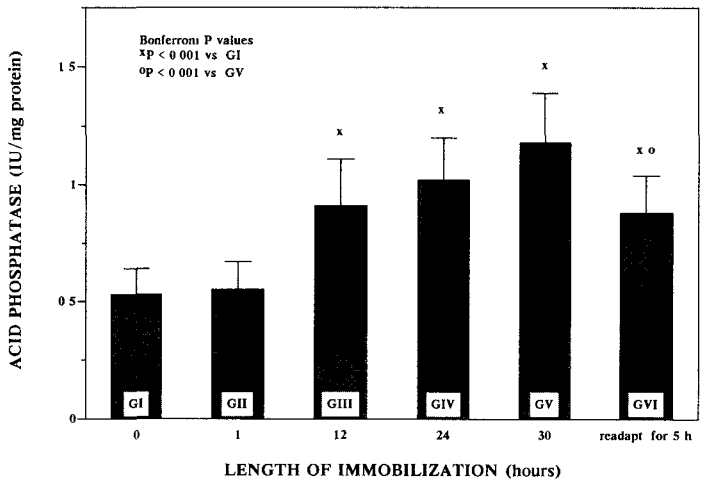

Figure 5. Lysosomal marker enzyme acid phosphatase in the lungs of rats subjected to immobilization stress.

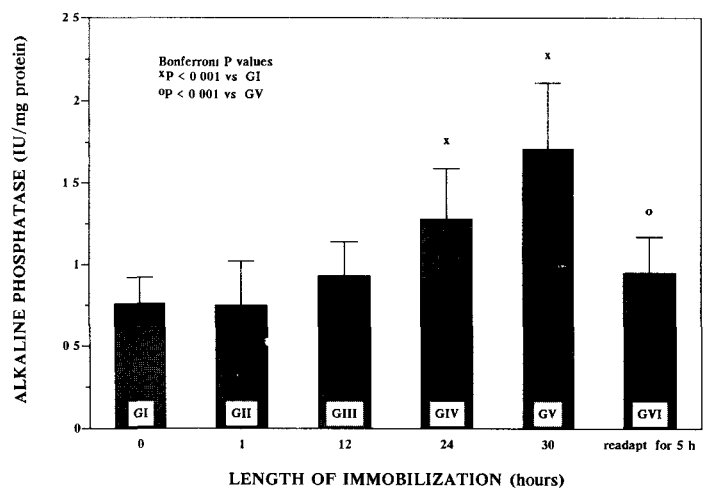

Figure 6. Plasmatic membrane marker enzyme alkaline phosphatase in the lungs of rats subjected to immobilization stress.

phatase, are illustrated in Fig. 5. A significant increase in the enzyme activity was observed for immobilization time of 12 h ($P < 0.001$ vs. control group GI) and longer only. Maximum activity was observed for GV (immobilization time 30 h). Readaptation for 5 h after 30 h immobilization (GVI) led to a significant decrease

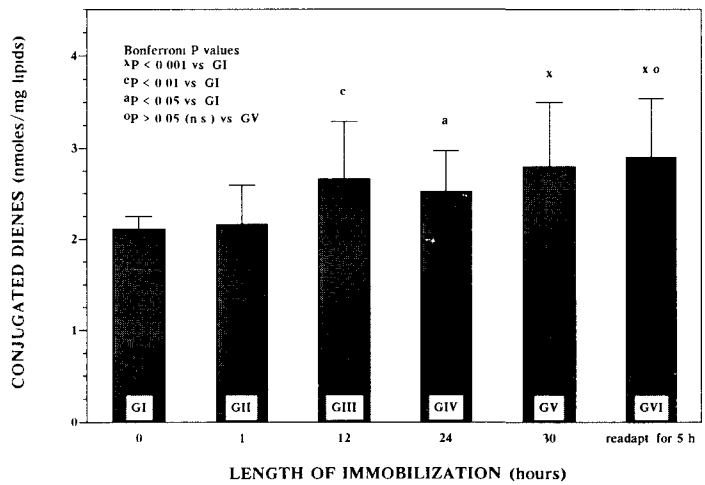

Figure 7. Conjugated dienes in the lungs of rats subjected to immobilization

of acid phosphatase activity ($P < 0.001$ vs. GV).

The marker enzyme alkaline phosphatase also increased its activity as a result of immobilization stress (Fig. 6). Significant changes were observed, however, for immobilization time of 24 h ($P < 0.001$ vs. GI) and longer. In addition, a significant decrease ($P < 0.001$ vs. GV) in the enzyme activity was measured after 5 h of readaptation (GVI).

All these findings suggested that significant destruction of the alveolo-capillary membrane occurs after immobilization for at least 24 h. Shorter times of immobilization (12 h) are associated with the activation of lysosomal hydrolases but without significant alterations of the plasmalemma.

Conjugated dienes and fluorescent products of lipid peroxidation

Lipid peroxidation is a complex process, including the generation of initial products such as conjugated dienes and fluorescent end products (Halliwell and Gutteridge 1989). Our further experiments were focused on changes of these two kinds of products. Fig. 7 and Fig. 8 illustrate the main results of these studies. Even a 12 h immobilization stress was found to cause significant elevations of both, conjugated dienes and fluorescent products of lipid peroxidation. Progressive rise of the levels of fluorescent products of lipid peroxidation was recorded upon increasing the immobilization time. This trend did not reach saturation even after 30 h of immobilization and continued during the readaptation stage ($P < 0.001$, GVI vs. GV). It is interesting to note that conjugated dienes, virtually, did not further increase either with the longer times of immobilization or after the time of readaptation studied.

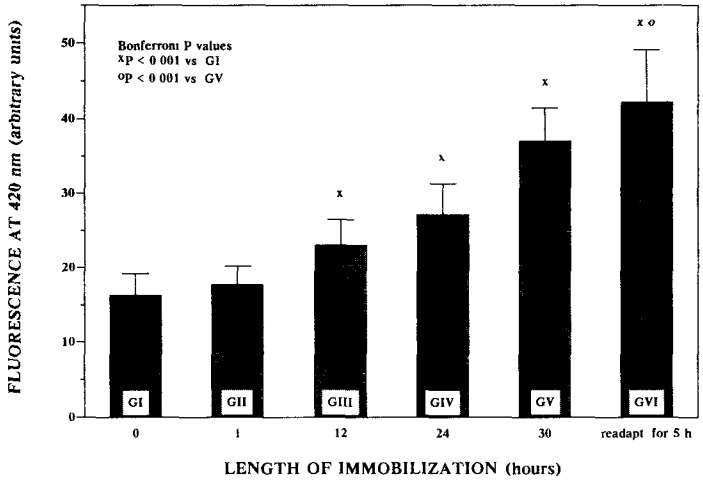

Figure 8. Fluorescent products of lipid peroxidation in the lungs of rats subjected to immobilization

Table 1. Contents of polyunsaturated fatty acids in phospholipids extracted from the lungs of rats subjected to immobilization (in percentages)

Length of immobilization (hours)+	Linoleic acid (C18 2)	Arachidonic acid (C20 4)
0 (GI)	3 86 ± 0 56	3 50 ± 0 61
1 (GII)	2 71 ± 0 10[a]	1 51 ± 0 26[a]
12 (GIII)	2 65 ± 0 16[a]	2 20 ± 0 14[a]
24 (GIV)	3 01 ± 0 33[a]	2 57 ± 0 33[a]
30 (GV)	2 65 ± 0 19[a]	2 66 ± 0 49[a]
30 (GVI) (+5 h readaptation)	2 51 ± 0 19[a]	1 02 ± 0 35[a b]

+15 rats in each group
Bonferroni P value $^{a}P < 0\ 001$ vs GI, $^{b}P < 0\ 001$ vs GV

Changes in fatty acid composition of phospholipids

As is well known, the main substrate of lipid peroxidation in the membrane is polyunsaturated fatty acids of the phospholipids Therefore, our subsequent experiments were designed to investigate immobilization stress-induced changes of fatty acid composition of phospholipids Table 1 and Table 2 summarize the results obtained Table 1 gives the concentrations of linoleic (C-18 2) and arachidonic (C-

Table 2. Contents of saturated and monounsaturated fatty acids in phospholipids extracted from the lungs of rats subjected to immobilization (in percentages)

Length of immobilization (hours)[+]	Saturated fatty acids[*]	Monounsaturated fatty acids[**]
0	73.21 ± 1.77	19.74 ± 0.91
1	74.87 ± 0.87	20.52 ± 0.70
12	76.28 ± 1.41[a]	19.16 ± 1.04
24	74.32 ± 1.60	19.05 ± 1.02
30	74.10 ± 1.82	20.90 ± 1.74
30 (+5 h readaptation)	75.36 ± 1.10[b]	20.48 ± 1.51

[+]15 rats in each group.
[*]Saturated fatty acids include C10:0; C12:0; C14:0; C15:0; C16:0; C17:0 and C18:0.
[**]Monounsaturated fatty acids include C14:1; C16:1; C17:1 and C18:1.
Bonferroni P value: [a]$P < 0.001$ vs. GI; [b]$P < 0.01$ vs. GI.

20:4) fatty acids, the major substrates of lipid peroxidation. Significant decreases in the concentrations of both fatty acids were observed for the alarm stage. The concentrations of these fatty acids remained diminished during the resting stages. The arachidonic acid levels decreased during the readaptation stage again. Changes in the relative shares (given in percentages) of monounsaturated and saturated fatty acids in lung phospholipids are shown in Table 2. The data revealed a striking predominance of saturated fatty acids (C-10:0, C-12:0, C-14:0, C-15:0, C-16:0, C-17:0, C-18:0), accounting for nearly three quarters (73.21%) of the total fatty acids content of phospholipids. Monounsaturated fatty acids (C-14:1, C-16:1, C-17:1, C-18:1) constituted 19.74% while polyunsaturated fatty acids (C-18:2, C-20:4) were less than 8%. As is well seen from the Table, monounsaturated fatty acids remained almost unchanged during all stages of the immobilization stress. Slight increases in the concentrations of saturated fatty acids were observed but statistical significance was only reached for the stages of alarm and readaptation. In accordance with these results, a sustained, statistically significant increase of the important saturated/polyunsaturated fatty acids ratio was observed for all immobilization times studied.

Changes of vitamin E content

The key role of vitamin E in the protection of biological membranes against oxidation is well established. Obviously, a decrease of the vitamin E content in a given biological tissue creates better conditions for the initiation and development of lipid peroxidation. Therefore, our subsequent experiments were designed to study immobilization stress-induced changes of the vitamin E contents in the lungs (Fig. 9).

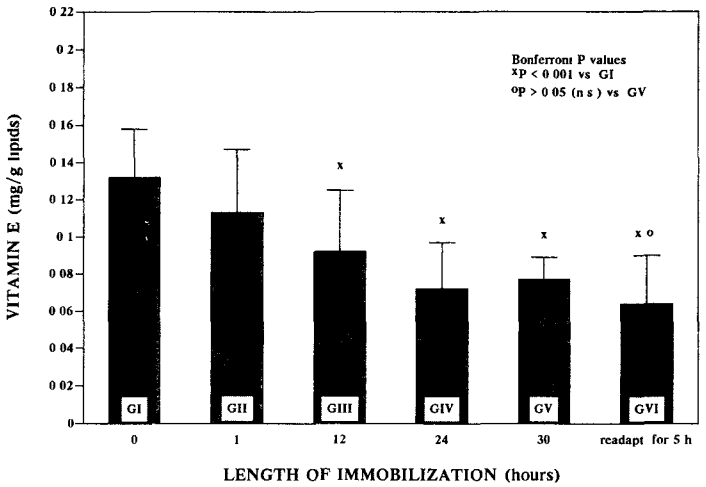

Figure 9. Vitamin E content in the lung tissue of rats subjected to immobilization

A significant decrease (by 30%, $P < 0\ 001$ vs GI) was found even for 12 h immobilization The vitamin E content was observed to further decrease with the increasing time of immobilization No significant decrease of vitamin E levels was observed for the readaptation stage ($P > 0\ 05$ vs GV) The content of vitamin E in the lungs of Group VI rats was half that found in the lungs of controls (GI)

Discussion

The ultrastructure of rat lungs after immobilization suggests that this type of stress causes progressive damage to the alveolar wall, with acute inflammatory reaction and development of oedema being the initial stages of this process Endothelial cells are affected first, followed by alveolar type I cells, and finally by alveolar type II cells The structural changes that occur in the lungs after 30 h of immobilization are similar to changes seen after some other forms of shock and stress, including the respiratory distress syndrome in human infants (Harrison et al 1969, Adamson et al 1970, Ratliff et al 1970, Moss 1972, Moss et al 1972, Connell et al 1975, Caddell et al 1987) The findings that the levels of the plasmalemma-marker enzyme alkaline phosphatase increase, but only for immobilization times exceeding 24 h, suggests that significant destruction of the plasmatic membrane of lung cells occurs at the end of the adaptation stage For 12 h immobilization, there is an increase of the lysosomal marker enzyme acid phosphatase, but without measurable damage to the plasmalemma

It is interesting to note, however, that although there was no plasmatic membrane destruction after 12 h of immobilization there were significant rises in the

levels of both conjugated dienes and fluorescent products of lipid peroxidation. Results obtained in other experiments provide circumstantial evidence for increased lipid peroxidation at 12 h of immobilization. Thus, the decreased lung contents of polyunsaturated fatty acids observed even during the alarm stage, and the increased saturated/polyunsaturated fatty acids ratio during all stages of stress, can be attributed not only to changes of the lipid metabolism but also to abnormal intensification of lipid peroxidation.

On the other hand, it is well known that vitamin E is essential for the protection of the cell membrane against lipid peroxidation. Its antioxidant action is due either to reactions with the free radical intermediates of lipid peroxidation or to scavenging of oxygen-centred radicals (Slater 1984; Halliwell 1987; Yagi 1987; Kagan 1988; Halliwell and Gutteridge 1989). Therefore, the decreased content of vitamin E in the lungs of rats subjected to 12 h immobilization stress suggests that an intensive generation of free radicals takes place. Furthermore, as it has been shown earlier (Thomas et al. 1989), the observed 30% decrease of vitamin E levels seems to create good conditions for the initiation and development of lipid peroxidation in the lung tissue.

However, the mechanism underlying the induction of lipid peroxidation in lung cell membranes during immobilization stress remains unclear. Lipid peroxidation may be triggered by the stress-induced high levels of plasma catecholamines (Kvetňanský and Mikulaj 1970; Selye 1976; Haggendal et al. 1987); the release of transition metal complexes from various storage sites (Packer 1985; Halliwell and Gutteridge 1989); the development of hypoxia and metabolic acidosis (Poyarov et al. 1990); etc. On the other hand, the intact structure of biomembranes is considered to be a safe protection against lipid peroxidation (Slater 1984; Halliwell 1987; Yagi 1987; Kagan 1988; Halliwell and Gutteridge 1989). It can therefore be not ruled out that the observed lipid peroxidation is due to changes in the membrane architecture resulting from some non-oxidative mechanism. Our data, however, suggest that lipid peroxidation possibly develops before the appearance of any measurable destruction of the plasmatic membranes. This fact might mean that in our case, lipid peroxidation is the cause, rather than the consequence of the lung structure damage.

Further evidence on the role of lipid peroxidation in the destruction of lung cell membranes induced by immobilization stress can be obtained by investigating changes of the morphological and biochemical parameters studied in this work, but under conditions of vitamin E overload or deficiency. Such experiments are under way in our laboratory.

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