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Effects of simvastatin treatment on oxidant/antioxidant state and ultrastructure of streptozotocin-diabetic rat lung

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In the present study, we investigated the effects of simvastatin, a 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor, on lipid metabolism, lipid peroxidation, antioxidant enzyme activities and ultrastructure of diabetic rat lung. Diabetes was induced by a single injection of streptozotocin (45 mg kg^{-1}, i.p.). After 8 weeks induction of diabetes, some control and diabetic rats were treated with simvastatin (10 mg kg^{-1} rat day^{-1}; orally) for 4 weeks. Diabetes resulted in significantly high levels of blood glucose and plasma lipids. Malondialdehyde levels were unchanged after 12-week-old diabetic rats, whereas catalase activity significantly decreased in the lung. Glutathione peroxidase activity and nitric oxide level were significantly elevated in the diabetic lung. Histological analysis of the diabetic lung revealed some deterioration in the structure. Simvastatin treatment reduced plasma lipid levels and partially decreased the severity of hyperglycaemia. Catalase, glutathione peroxidase activities and nitric oxide levels were partially restored and accompanied by improved structure in diabetic lung by the simvastatin treatment. These results suggest that structural disturbances and alteration of antioxidative enzyme activities occurred in diabetic lung. Simvastatin treatment may provide some benefits in the maintenance of antioxidant status and structural organization of diabetes-induced injury of lung. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS — catalase; glutathione peroxidase; lung; malondialdehyde; simvastatin; STZ-diabetes

INTRODUCTION

Reactive oxygen species (ROS) are well characterized mediators of cell and tissue injury.[1] Generally, cells defend themselves from ROS and other toxic oxygen species by a variety of mechanisms including nonenzymic and enzymic defence systems.[2] An ineffective scavenging capacity of antioxidant systems may play an important role in determining the degree of oxidative stress.[3] The diabetic state itself causes oxidative stress via autooxidation of glucose, protein glycation and due to increased lipid peroxidation through quantitative and qualitative changes in lipid metabolism.[4,5]

The lung contains a glucose transport system that is stimulated by insulin, so that diabetes may have effects on glucose utilization and lead to disturbances of the lung.[6] It is known that diabetes causes mild functional abnormalities and some structural modifications in pneumocytes but investigations of the role of diabetes-induced oxidative stress on the pulmonary system are few.[7,8]

Simvastatin is a 3-hydroxy-3-methyl-glutaryl (HMG-CoA) reductase inhibitor which is widely used in cholesterol-lowering therapy including diabetic patients.[9] HMG-CoA reductase inhibitors inhibit both synthesis of cholesterol and the synthesis of isoprenoids from mevolanate, which have an essential role in proliferation, migration and signalling of cells.[10] Furthermore the possible antioxidant role of HMG-CoA reductase inhibitors has also been studied in the cardiovascular system but it is not clearly known in lung. Thus we designed experiments to examine (i)

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the effect of diabetes on the ultrastructural components and oxidant/antioxidant status of the lung and (ii) to determine the effect of simvastatin treatment on these parameters of the diabetic lung.

MATERIALS AND METHODS

Male Wistar rats weighing 200–250 g were fed a standard rat chow diet and had access to water *ad libitum.* The experiments were performed in accordance with the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985, revised version). At the beginning of the experimental period the rats were divided into controls and diabetics. Diabetes was induced by a single injection of streptozotocin (STZ, 45 mg kg^{-1}, i.p.). Two days after the STZ injection, blood glucose levels were determined in blood samples collected from the tail vein. The rats with blood glucose levels higher than 250 mg dl^{-1} were accepted as diabetic. Eight weeks after induction of diabetes, some control and diabetic animals were treated with simvastatin (10 mg kg^{-1} day^{-1} orally) for 4 weeks. At the end of the experimental period (12 weeks), rats were anaesthetized with urethane (30%, i.p.) and were sacrificed. Blood samples were drawn by cardiac puncture and lungs removed by opening the thoracic cavity.

Blood measurements

Blood glucose concentrations were measured by an Ames glucometer (Glucometer III, Bayer Diagnostics, France). Plasma total cholesterol and triacylglycerol levels were determined with a commercially available enzyme kit (Wako, Osaka, Japan).

Tissue measurements

Lungs were immediately washed with ice-cold saline and stored at −20°C until analysis. At the time of analysis lung tissues were homogenized for determining oxidant and antioxidant status. Catalase (CAT) activity was determined according to the method of Aebi.[11] The CAT-mediated decomposition of H_2O_2 was monitored spectrophotometrically at 240 nm. Glutathione peroxidase (GSH-Px) activity was measured according to Lawrence and GSH-Px activity was expressed as μmol of NADPH oxidized to NADP min^{-1} mg^{-1} lung protein.[12]

Malondialdehyde (MDA) levels were measured by the fluorometric method of Yagi.[13] Protein levels of lung tissue were determined by the method of Lowry *et al.*[14] Nitric oxide (NO) levels were measured by the method previously described.[15]

Microscopy studies

Lung tissues from all animals in the study were fixed in a solution of 2.5% glutaraldehyde in a phosphate buffer at pH 7.2 for 2–4 h and outlined with 1% osmium tetroxide. Later the materials were dehydrated in graded ethanol solutions and embedded in araldite. For each sample eight to 10 specimens were analysed. Selected sampled were sectioned with a Leica Ultracut R ultramicrotome, stained with uranyl acetate and lead citrate and then analysed with an electron microscope (Leo 906-E transmission electron microscope).

Drugs and statistical analysis

All chemicals used in experiments, except simvastatin, were purchased from Sigma Chemical (St. Louis, MO, USA). Simvastatin was manufactured by Raubaxy Lab. (New Delhi, India). The results are expressed as mean ± SEM. Statistical analysis of data was performed using ANOVA followed by Student–Newman–Keuls test.

RESULTS

Metabolic parameters

Blood glucose levels were significantly elevated in both simvastatin-treated and the untreated diabetic groups when compared to both control groups. Simvastatin treatment partially reduced the hyperglycamia compared with the untreated diabetics. Increased plasma cholesterol and triacylglycerol levels were restored by simvastatin treatment in diabetics. These parameters were not affected by simvastatin treatment in the control group (Table 1).

Changes in lipid peroxidation levels and antioxidant capacity of lung

The oxidative state of lung was analysed by determining MDA equivalents. The MDA levels of the lung were not different among the groups (Table 2). Decreased CAT activity was reversed by simvastatin treatment in the diabetic group. Increased activity of GSH-Px in the diabetic group was normalized by simvastatin. There was no significant difference in these parameters between simvastatin-treated and untreated control groups.

Table 1. Blood glucose and plasma lipid levels at the end of the experiment in control (C), simvastatin-treated controls (CS), diabetic (D) and simvastatin-treated diabetic (DS) rats

	C ($n = 8$)	CS ($n = 8$)	D ($n = 11$)	DS ($n = 10$)
Blood glucose (mg dl^{-1})	$100 \pm 6.3^{\ddagger}$	$112 \pm 4.7^{\ddagger}$	413 ± 6.8*	$273 \pm 9.3^{*,\ddagger}$
Plasma triacylglycerols (mg dl^{-1})	$85 \pm 7.5^{\ddagger}$	$89 \pm 7.2^{\ddagger}$	154 ± 5.9*	$120 \pm 7.5^{*,\dagger}$
Plasma cholesterol (mg dl^{-1})	$65.25 \pm 4.5^{\ddagger}$	$60 \pm 3.1^{\ddagger}$	125 ± 9.2*	$74 \pm 6.4^{\ddagger}$

Results are expressed as mean ± SEM. n, number of animals.
*$p < 0.001$ vs. control; $^{\dagger}p < 0.01$; $^{\ddagger}p < 0.001$ vs. diabetic animals.

Table 2. Lipid peroxidation and antioxidant state of lung in control (C), simvastatin-treated control (CS), diabetic (D) and simvastatin-treated diabetic (DS) rats

	C ($n = 6$)	CS ($n = 8$)	D ($n = 10$)	DS ($n = 10$)
MDA (nmol mg^{-1}protein)	0.127 ± 0.009	0.128 ± 0.009	0.126 ± 0.007	0.125 ± 0.009
GSH-Px (μmol min^{-1} mg^{-1}protein)	$0.162 \pm 0.012^{\dagger}$	$0.156 \pm 0.009^{\dagger}$	0.187 ± 0.010*	$0.165 \pm 0.012^{\dagger}$
CAT (ks^{-1} mg^{1}protein)	$0.113 \pm 0.009^{\dagger}$	$0.111 \pm 0.006^{\dagger}$	0.090 ± 0.007*	$0.108 \pm 0.008^{\dagger}$
NO (μmol)	$42.480 \pm 3.196^{\dagger}$	$41.512 \pm 2.61^{\dagger}$	96.813 ± 5.922*	$52.687 \pm 2.892^{*,\dagger}$

Results are expressed as mean ± SEM. n, number of animals. MDA, malondialdehyde;
GSH-Px, glutathione peroxidase; CAT, catalase; NO, nitric oxide. *$p < 0.001$ vs. control; $^{\dagger}p < 0.001$ vs. diabetic.

NO levels of lung

Lung NO levels in the diabetic group were higher than among the other groups. Simvastatin treatment partially decreased NO levels in diabetic rats towards the control value (Table 2).

Investigation of ultrastructure of lung

Under electron microscopic investigations, the control group presented ultrastructure of lung that was normal and characteristic of the description in literature (Figure 1). Simvastatin treatment did not significantly affect the ultrastructure of lung in the control group (Figure 2). In the diabetic rats, various pathological changes in lung structure, such as congestion in the vessels and enlargement of alveolar space, increase in number of macrophages both in the alveolar space and in the interalveolar septum were visible in the micrographs. Collagen, elastin fibres were increased in the interalveolar septum, and some alveolar spaces and capillaries were collapsed (Figure 3a

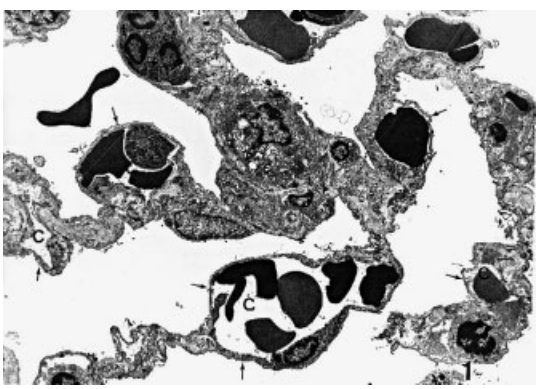


Figure 1. Electron micrograph of lung from control group. Interalveolar septa are normal in appearance. C, capillary, PII, type II pneumocyte; ↓, blood–air barrier. × 2156

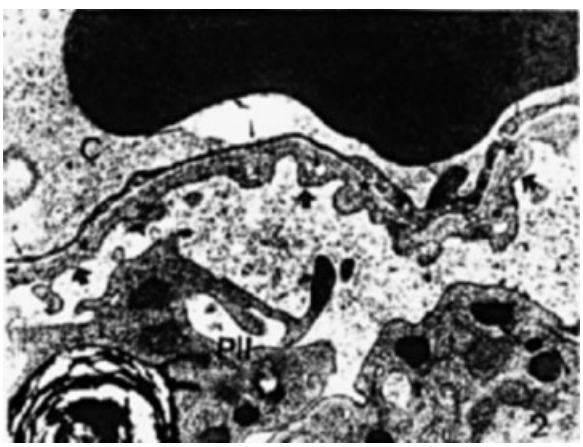
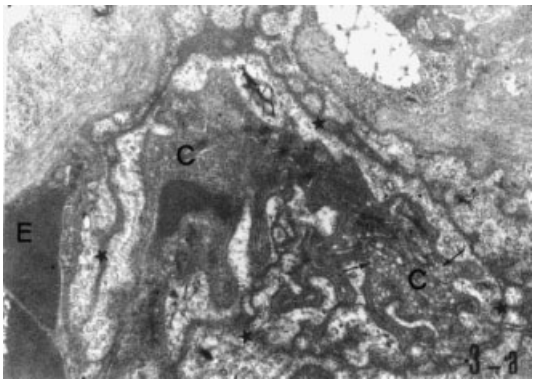


Figure 2. Electron micrograph of lung from simvastatin-treated control animal. The blood–air barrier is of normal appearance. C, capillary; PII, type II pneumocyte; ↓, endothelial cell; ⬇, type I pneumocyte; *, fused basal lamina. × 16 700



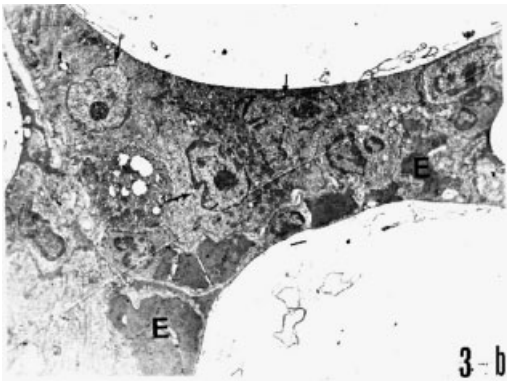


Figure 3. (a) Electron micrograph of lung from diabetic group. Over a large surface, the alveolar space (*) and some capillaries (C) become collapsed. Plasmalemmal vesicles (↓) are seen in the endothelial cytoplasm. E, erythrocytes in capillary lumen. × 12 930. (b) Electron micrograph of lung from diabetic group. Increased amounts of macrophages (↓↓↓) are seen in the thickened interalveolar septum. E, erythrocytes in capillary lumen. × 2156

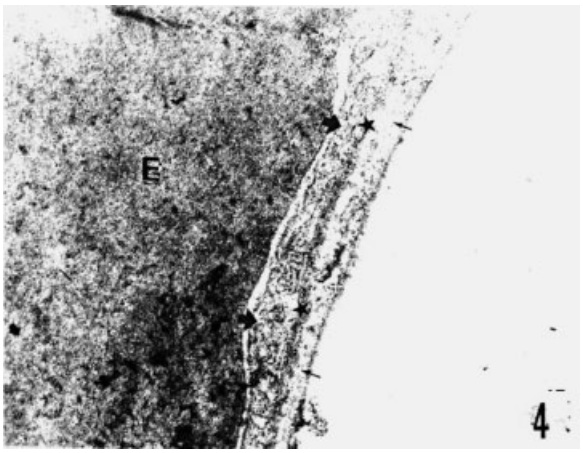


Figure 4. Electron micrograph of blood–air barrier from the simvastatin-treated diabetic group. Type I pneumocyte (↓) endothelial cytoplasm (↓), fused basal lamina (*) and erythrocytes (E) are nearly normal in appearance. × 27 800

and b). Simvastatin treatment reversed these alterations in lung tissue and the appearance became nearly normal (Figure 4).

DISCUSSION

In the present study, diabetes caused some alterations in antioxidant capacity and structural deterioration without a change in lung lipid peroxidation. Simvastatin treatment restored biochemical and structural disorders of diabetic lungs.

Diabetes is a systemic disease that produces some alterations in structural and functional complications in several organs such as kidney and heart. The aetiology of diabetic complications is uncertain. It has been proposed that several mechanisms and various factors act both independently and together to contribute to the development and progression of these complications. Oxidative stress, non-enzymic protein glycosylation and alteration of NO metabolism occur as a consequence of diabetes-related metabolic disorders.[16]

The thickenings of basal lamina are one of the most significant complications of diabetes that have been frequently observed in various diabetic tissues such as kidney, retina, and heart; it is the classical morphologic findings in diabetic microangiopathy.[17–19] In the progression of microangiopathy, increased nonenzymic glycation of proteins and peptides plays a major role and may be responsible for extracellular matrix accumulation.[18] In the present study, the electron microscopic examination of diabetic lung tissue showed that some capillary and alveoli had become narrowed. This may be another reason for the expanded interstitium and extracellular matrix. These alterations result in an increased thickness of the membrane that may inhibit gas transport by diffusion and cause a decreased oxygen supply to the tissue. In addition the occurrence of intravascular macrophages in the diabetic lung could be correlated with inflammatory reactions. Our findings are similar to the results of many authors who described a thickening of alveolar epithelial and pulmonary capillary basal lamina in diabetic subjects and experimental diabetes.[20,21] Ofulue and Thurlbeck postulated that the thickened alveolar and vascular basal lamina might be due to the increased lysloxidase activity, which plays a vital

role in the formation of connective tissue.[20] It has been reported that the products of oxidative modification of proteins and carbohydrates were increased via these enzymes and were accumulated in diabetic patients and the STZ-diabetic rat.[20,21]

In this study, our results showed that there is an increase in NO concentrations of diabetic lung tissue. This may be due to the increased incidence of infections in the lung. Inflammatory diseases of the respiratory tract such as asthma and acute respiratory distress syndrome are commonly characterized by an increased production of NO within respiratory epithelial and inflammatory-immune cells and markedly elevated local production, presumably as an additional host defence mechanism against infections.[22]

The other data of the present study indicate that diabetes caused alterations of the antioxidant enzyme capacity of the lung without any change in the MDA levels. The mechanisms of how MDA was kept at normal levels contrary to reduction in CAT activity and augmentation in GSH-Px, is not clear. It seems that the lung of the diabetic rat has partly increased resistance to peroxidative stress. Perhaps the alteration in the antioxidant enzyme and non-enzymic system capacities to scavenge the increased ROS produced, is sufficient in diabetic lung. Any excess production of H_2O_2 can be detoxified by both CAT and GSH-Px in a concentration-dependent manner.[2] CAT is involved in the detoxification of high H_2O_2 concentrations whereas GSH-Px is sensitive to lower concentrations. Doroshow *et al.* reported that in tissues lacking CAT activity H_2O_2 detoxification is critically dependent on the activity of GSH-Px.[23] In this regard, the increased activity of GSH-Px indicated a compensation for decreased CAT activity in diabetes.

We showed that simvastatin treatment decreased plasma lipids and provided a relatively better glucose metabolism in diabetic animals. These effects of simvastatin in this study parallel previous studies.[24,25] Also simvastatin treatment reversed antioxidant enzyme capacity and ultrastructure in the diabetic lung. This beneficial effect of simvastatin is multifactorial. Firstly, it may improve the biochemical structure of diabetic lung. It has been shown that in diabetic pulmonary tissue, triacylglycerols and non-esterified fatty acids were increased whereas total lipid content was unchanged when compared to the control rats.[26] It has been reported that simvastatin reduces plasma cholesterol and free cholesterol concentrations in liver and lung culture.[27] Thus simvastatin treatment may reorganize the lipid profile of diabetic lung. Secondly simvastatin may reduce the thickening of basal laminae via inhibition of the synthesis of isoprenoids. It has been shown that treatment with HMG-CoA reductase inhibitors can reduce the extent of thickening of atherosclerotic plaque, cardiac hypertrophy and excessive deposition of extracellular matrix in diabetic nephropathy.[28–30] In addition simvastatin inhibited the rate of growth of human bronchial smooth muscle cells in a concentration-dependent manner *in vitro*.[31] In our study, we demonstrated that simvastatin treatment improves the ultrastructure of lung especially the thickening of alveolar and capillary basal lamina in diabetic lung. This result could be partially attributed to an effect on smooth muscle. Simvastatin is able both to normalize the greatly increased viscosity in the microcirculation and alter gas exchange parameters in hyperlipidaemic patients.[32,33] On the other hand the reduced level of NO is responsible for the improvement in the diabetic lung. It has been shown that iNOS expression is markedly reduced by simvastatin in association with a reduction in intima thickening in atheriosclerosis.[34] The reduction in iNOS gene expression can attenuate the oxidative stress, which is associated with NO metabolites especially peroxynitrates and nitronium ions that appear in the presence of oxygen radicals. Therefore the reduction in NO levels may indicate a reduced stimulation of cells. Thirdly simvastatin may have an antioxidant effect in lung as well as in vascular tissue.[35] It is the first report that simvastatin alters the activity of CAT and GSH-Px in diabetic lung.

In conclusion, we have found that simvastatin can induce significant prevention of damage of the ultrastructure of diabetic lung by improving the antioxidant capacity and NO levels.

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