

Effects of Simvastatin Treatment on Oxidant/Antioxidant State and Ultrastructure of Diabetic Rat Myocardium

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Abstract. In the present study we investigated the effects of simvastatin treatment on lipid metabolism and peroxidation, antioxidant enzyme activities and ultrastructure of the diabetic rat myocardium. Diabetes was induced by single injection of streptozotocin (45 mg/kg i.p.). Eight weeks after induction of diabetes, a subgroup of control and of diabetic rats was treated with simvastatin for 4 weeks (10 mg/kg/day, orally). Blood glucose, plasma cholesterol and triacylglycerol, as well as levels of cardiac thiobarbituric acid reactive substances (TBARS) were significantly increased in diabetic rats. The activities of antioxidant enzymes, catalase (CAT) and glutathione peroxidase (GSHPx), were also elevated in the diabetic myocardium. Treatment with simvastatin markedly reduced serum triacylglycerol and cholesterol, and partially controlled hyperglycemia in diabetic animals. The increased activation of antioxidant enzymes and the excess of lipid peroxidation measured by TBARS were completely reversed by simvastatin treatment. Diabetic rats displayed ultrastructural ischemia-like alterations of cardiomyocytes and capillaries, which support oxidative stress-induced tissue remodelling. In the diabetic myocardium simvastatin treatment partly attenuated angiopathic and atherogenic processes, detected by electron microscopy. These results suggest that simvastatin, known as a lipid-lowering drug, may positively affect diabetes induced cardiovascular complications *via* reducing risks of atherosclerotic pathological processes, such as imbalance between oxidant and antioxidant state.

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Introduction

Metabolic derangements have been suggested to be important risks for development of cardiovascular diseases in diabetes mellitus (Kannel and McGee 1994; Goldberg 2000). In diabetic patients, metabolic abnormalities may lead to hypertension, coronary atherosclerosis and heart attack resulting in congestive heart failure (Ferrannini et al. 1996; Herlitz and Malmerg 1999). Among the molecular mechanisms proposed for cardiovascular complications of diabetes mellitus, oxidative stress seems to be a crucial factor (Baynes and Thorpe 1999; Kowluru et al. 2000). As well known, hyperglycemia generates abnormally high levels of reactive oxygen species (ROS) and compromises natural antioxidant defense systems (Hunt et al. 1990; Kakkar et al. 1996; Mak et al. 1996). ROS exert oxidative effects on plasma lipoproteins, membrane lipids and enzyme proteins, changing their function and thus inducing atherosclerosis and impaired cardiac performance (Chisolm et al. 1992). The alterations in the activities of antioxidant enzymes may also influence the susceptibility of the cardiovascular system to oxidative stress induced cytotoxicity (Kakkar et al. 1996). In accordance with other laboratories, we showed that antioxidant supplementation reduced some diabetes-induced metabolic and functional abnormalities (Karasu et al. 1997; Koçak et al. 2000) in the cardiovascular system (Kaul et al. 1996; Stefek et al. 2000; Zobali et al. 2002) *via* improving the prooxidant feature of diabetes and the redox status of cells. Moreover, dyslipidemia alone may be the reason for enhanced levels of thiobarbituric acid reactive substances (TBARS) in diabetics since previous studies directly linked hyperlipidemia with increased serum and tissue concentrations of lipid peroxidation products (De La Cruz et al. 2000). Previously, we demonstrated that the lipid peroxidation inhibiting effect of gemfibrozil, an antihyperlipidemic drug, depended largely on its effect on circulating lipids without significant changes in glucose metabolism and antioxidant enzyme activities in diabetic rats (Ozansoy et al. 2001).

Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, was shown to produce a significant reduction in coronary heart disease by the reducing plasma cholesterol (Scott 1997). The clinical benefits of HMG-CoA reductase inhibitors in heart disease are far greater than would be expected from simply lowering plasma cholesterol (Vaughan et al. 1996). The beneficial effects of simvastatin can also be attributed to its other regulatory properties affecting endothelial function, platelet aggregation, coagulation and lipid peroxidation (Jorge et al. 1997; O'Driscoll et al. 1997).

The aim of the present study was to investigate whether the changes in oxidative metabolism and ultrastructure of the heart could be reversed by oral simvastatin treatment in experimental diabetes.

Materials and Methods

Animals and treatment protocols

Male Wistar rats weighing 200–230 g were housed under constant climatic conditions during the 12-week experimental period beginning 48 h after either vehicle or streptozotocin (STZ) injection. The diets and water were given ad libitum to all animals. The experimental groups comprised control ($n = 8$), simvastatin-treated control ($n = 10$), diabetic ($n = 10$), simvastatin-treated diabetic ($n = 12$) rats. Diabetes was induced by a single injection of STZ (45 mg/kg body weight, i.p.) to animals fasted overnight. Two days after STZ administration, the animals with glucose level 250 mg/dl or more were considered diabetic and were included in the study. Eight weeks after injection of STZ, a subgroup of the control and the diabetic rats were started on simvastatin treatment (10 mg/kg/day in 0.5 % carboxymethylcellulose solution, orally) for 4 weeks.

Blood and tissue analysis

Blood glucose concentrations were measured by an Ames glucometer (Glucometer III, Bayer Diagnostics, France).

Total plasma cholesterol and triacylglycerol concentrations were determined by commercially available enzyme kits (Wako, Osaka, Japan).

Myocardial tissue homogenates were used for measurement of TBARS levels as an index of lipid peroxidation, according to the previously described fluorimetric method of Yagi (1987). Tissue homogenate (200 μ l) was heated for 60 min at 95°C together with 250 μ l of 42 mmol/l 2-thiobarbituric acid and 750 μ l of 0.19 mmol/l H_3PO_4 . Standard samples were prepared from malondialdehyde-bis-diethylacetal. The samples were precipitated with a mixture of methanol and 1 mol/l NaOH and centrifuged at 4000 rpm for 5 min. Fluorescence was then measured in the supernatant, the excitation and emission wavelength was 532 nm and 553 nm, respectively.

The activity of catalase (CAT) was measured spectrophotometrically by the method of Aebi (1983). Cleaned and minced ventricles were homogenized in 3 volumes of 50 mmol/l phosphate buffer (pH 7) with Triton X-100. Twenty μ mol/l of homogenate was added to a cuvette containing the phosphate buffer to achieve the final volume of 2.0 ml. The reaction was started by addition of 1.0 ml of 30 mmol/l H_2O_2 . The rate of H_2O_2 decomposition was determined at 20°C against a blank containing the prepared enzyme solution but no substrate at the absorbance of 240 nm. The activity of tissue is expressed as k/s/mg protein, where k is the first order rate constant.

The activity of glutathione peroxidase (GSHPx) was measured using the method of Lawrence and Burk (1976). Total protein in homogenates was determined by the method of Lowry et al. (1951). In this method, GSHPx activity is coupled to NADPH utilization, and the production of $NADP^+$ was measured spectrophotometrically at 340 nm. The assay mixture consisted of 76 mmol/l phosphate buffer

with EDTA and NaN_3 (pH 7), 0.150 mg $10,000 \times g$ supernatant protein of tissue, 0.1 mmol/l NADPH, 4.0 mmol/l glutathione (GSH) and 1.5 U glutathione reductase in the final volume of 500 μl . The reaction was started by addition of 3.0 mmol/l H_2O_2 . GSHPx activity was expressed as μmol of NADPH oxidized to NADP^+ /min/mg protein for tissue.

Electron microscopy

Myocardial tissue was processed for electron microscope examination according to previously described techniques (Karasu et al. 1997). After isolation, ventricular samples were cut into small pieces, fixed at 4°C for 2 h in 2.5% buffered glutaraldehyde, and postfixed for 1.5 h with osmium tetroxide. Tissues were dehydrated in ethyl alcohol followed by propylene oxide and embedded in araldite. Ultra-thin circumferential sections (at least five sections were taken from three different levels of each specimen) were cut using a diatome knife (Agar Scientific, England), then mounted on a 3.05-mm, 20-mesh copper grid and contrast stained with saturated aqueous uranyl acetate for 30 min and Reynolds lead citrate for 5 min. Sections were examined using an electron microscope (LEO 906 E).

Drugs and statistical analysis

Except simvastatin, other chemicals used in the experiments were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Simvastatin was produced in Reubaxy Laboratory (New Delhi, India). The results are expressed as means \pm S.E.M. Statistical analysis was performed by using ANOVA followed by Student-Newman-Keuls test.

Table 1. Blood glucose, plasma lipids and antioxidant enzyme activities in myocardium of control (C), simvastatin-treated control (C+S), diabetic (D), simvastatin-treated diabetic (D+S) rats

	C (n = 8)	C+S (n = 10)	D (n = 10)	D+S (n = 12)
Blood glucose (mg/dl)	108 \pm 4	110 \pm 4	410 \pm 8*	276 \pm 13*†
Plasma triacylglycerol (mg/dl)	81 \pm 7	82 \pm 8	159 \pm 8*	127 \pm 8*#
Plasma cholesterol (mg/dl)	63.2 \pm 3.0	59.0 \pm 2.8	127.0 \pm 8.2*	72.5 \pm 3.4†
CAT ($\text{ks}^{-1} \cdot \text{mg}^{-1}$ protein)	0.027 \pm 0.004	0.030 \pm 0.003	0.062 \pm 0.005*	0.032 \pm 0.005
GSHPx ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein)	0.176 \pm 0.009	0.179 \pm 0.010	0.235 \pm 0.008*	0.181 \pm 0.008
TBARS ($\text{nmol} \cdot \text{mg}^{-1}$ protein)	0.054 \pm 0.003	0.056 \pm 0.004	0.071 \pm 0.003*	0.059 \pm 0.004

CAT, catalase; GSHPx, glutathione peroxidase; TBARS, thiobarbituric acid reactive substance. Values are expressed as mean \pm S.E.M. * $p < 0.001$ vs. control; † $p < 0.001$, # $p < 0.01$ vs. diabetic.

Results

All biochemical parameters determined in the study are represented in Table 1. Diabetes resulted in high blood glucose concentration and simvastatin treatment partially controlled the severity of hyperglycemia. Plasma cholesterol and triacylglycerol concentrations were markedly elevated in untreated diabetic rats. Simvastatin treatment significantly, but not completely, reversed the abnormalities in triacylglycerol concentrations and normalized the concentration of cholesterol in plasma of diabetic rats. Plasma glucose, cholesterol and triacylglycerol concentrations of control rats were not affected by simvastatin. The findings of TBARS measurements showed an excess of lipid peroxidation in the myocardium after 12 weeks of diabetes. STZ-diabetes led to an increase in the activity of cardiac CAT and GSHPx. Simvastatin treatment almost completely reversed lipid peroxidation, CAT, and GSHPx activities in the diabetic myocardium (Table 1).

In electron microscopic analysis, the control myocardium was characterized by normal architecture of cardiomyocytes. Mitochondria contained regular cristae (Figure 1). In simvastatin-treated control rats, the structure of the myocardium showed an organization similar to that observed in untreated control rats (Figure 2). Severe ultrastructural alterations were observed in cardiomyocytes of diabetic animals. There were disarrangement of myofilament bundles, loss of myofilaments and their partial fragmentation (Figure 3). Due to diabetes, there were changes in the thickness of glycocalyx and basal membrane (Figure 4). Myelin structures and increased amount of lipid droplets were seen in the vicinity of some mitochondria.

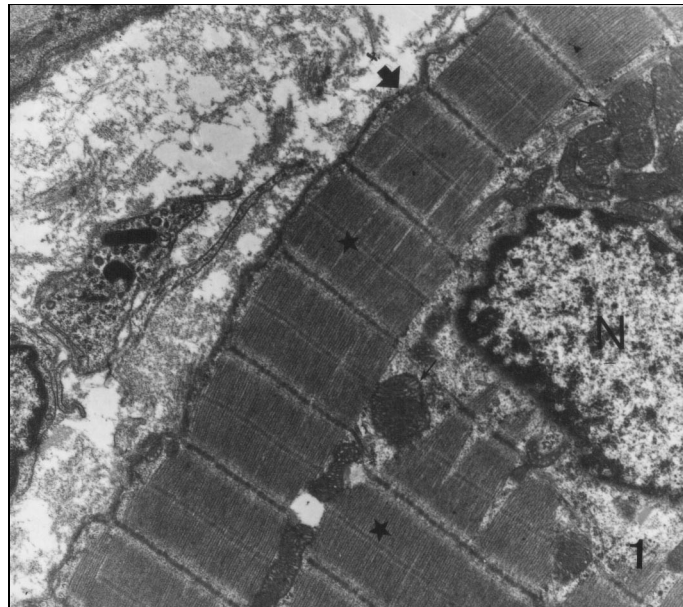


Figure 1. Control group. Parallel arrays of myofilaments (stars), regular structure and size of mitochondria (thin arrows) and glycocalyx (thick arrow) in a cardiomyocyte. N, nucleus (X 7750).

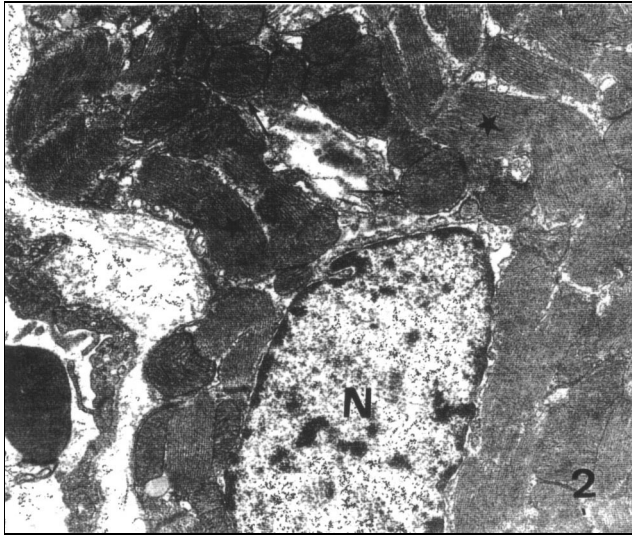


Figure 2. Simvastatin-treated control group. Similar aspect of control cardiomyocytes. Mitochondria (thin arrows), myofilaments (stars). N, nucleus (X 6000).

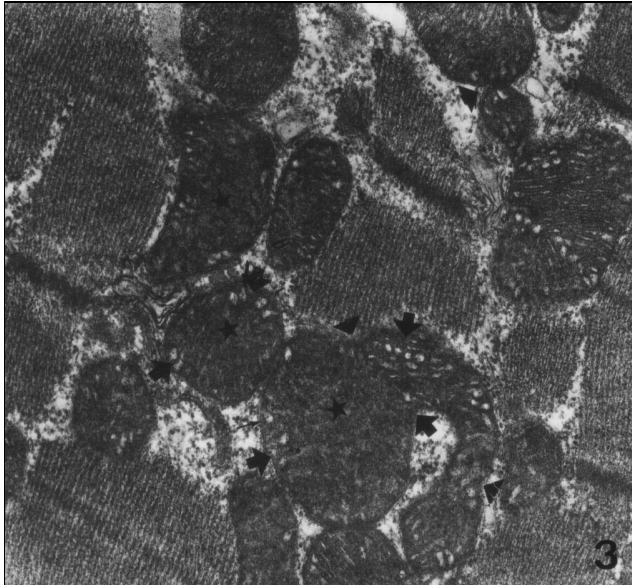


Figure 3. Diabetic group. Disorganisation of cristae (stars), widened intercrystal spaces (thick arrows), fusing mitochondria (arrow head) (X 21650).

Mitochondria were different in size and shape, showed disruption, loss of structural integrity, and the areas among cristae were enlarged and partially edematous (Figure 5). Endothelial cells contained a great amount of pinocytic vesicles and showed protrusions into the lumen. Endothelial cells of capillaries also exhibited structural alterations (Figure 6). The beneficial effect of simvastatin treatment was largely

Figure 4. Diabetic group. Varying size and shape of mitochondria. Some of them have vacuols (stars) and fusion with each other (thick arrows). Myelin structures (thin arrows) and lipid droplets (arrow head) are seen in the vicinity of some mitochondria (X 7750).

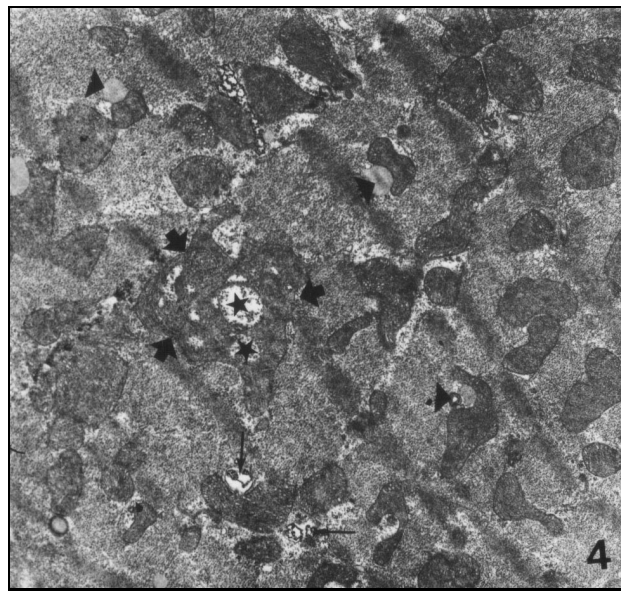
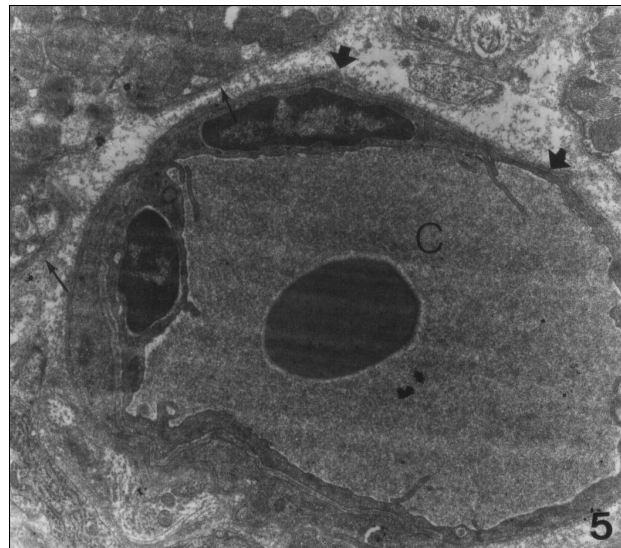


Figure 5. Diabetic group. A degenerated capillary (C), basal lamina (thick arrow) and glycocalyx (thin arrows) of diabetic myocardium (X 7750).



observed on myocytes. Parallel arrangements of myofilaments and practically normal shape of cristae and mitochondria were observed, while the presence of lipid droplets was less frequent (Figure 7). Intracellular edema, atherosclerotic lesions and ischemia-like injuries were markedly attenuated in simvastatin-treated diabetic animals.

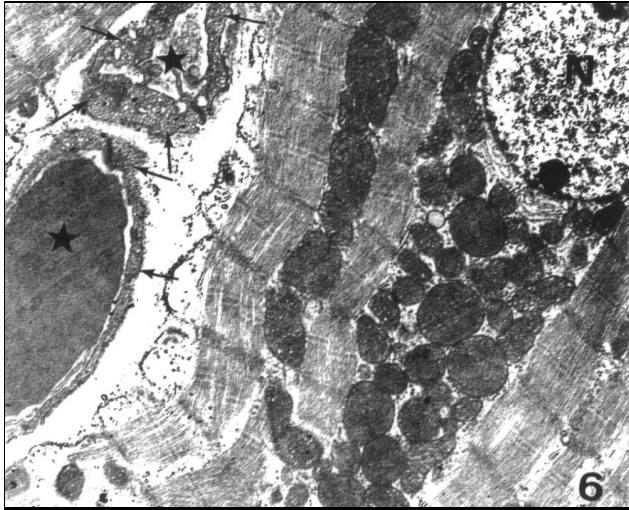


Figure 6. Diabetic group. Endothelial cell cytoplasm (arrows) of vessels (stars) contains a large number of pinocytotic vesicles. N, nucleus (X 6000).

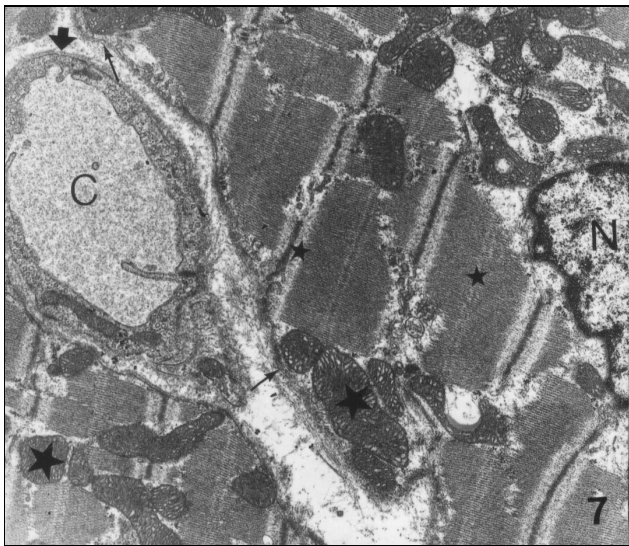


Figure 7. Simvastatin-treated diabetic group. Basal lamina (thick arrow), glycocalyx (thin arrow), mitochondria (big star), myofilaments (small star). N, nucleus (X 7750).

Discussion

In this study, STZ-diabetes was characterized by dyslipidemia, elevated plasma concentrations of triacylglycerol and total cholesterol, which is in agreement with results of other authors (Hunt et al. 1990; Chisolm et al. 1992; Kakkar et al. 1995; Karasu et al. 1997; Koçak et al. 2000; Stefek et al. 2000; Zobali et al. 2002). This study also confirmed that STZ-diabetes was characterized by increased oxidative

stress and the activity of antioxidant enzymes in heart. In addition to this, ultrastructural remodeling was present in the diabetic myocardium, which may result in its impaired function (Koltai et al. 1984; Jackson et al. 1985; Fitzl et al. 1999; Stefek et al. 2000).

The present study showed that 4-week simvastatin treatment, partially controlled hyperglycemia, normalized the plasma concentration of cholesterol and reduced plasma triacylglycerols in diabetic rats. Simvastatin treatment also reversed abnormalities in antioxidant enzyme activities and reduced peroxidation of lipids in the diabetic myocardium. Simvastatin treatment partially improved atherosclerotic changes in cardiomyocyte structure in diabetic animals. The derangements in mitochondria, myofibril and capillary structures and membranes were markedly attenuated by simvastatin treatment. The beneficial effect of simvastatin observed in the ultrastructure of the diabetic myocardium seems to be related to its lipid lowering and antioxidant properties.

As reported by several authors, under *in vivo* conditions, oxidative stress may result in compensatory elevation in the activity of antioxidant enzymes (Kakkar et al. 1995, 1996; Mak et al. 1996; Koçak et al. 2000; Stefek et al. 2000; Ozansoy et al. 2001; Zobali et al. 2002). This is supported by the results of the present study showing increased activity of GSHPx and CAT in the diabetic myocardium, reflecting increased peroxidative stress. In fact, the production and biological availability of H_2O_2 , detoxified by CAT and GSHPx, was shown to be elevated in diabetes. Oxidation of fatty acids, as well as ROS production is associated with the membranes of cell organelles, such as mitochondria and endoplasmic reticulum. Our results showed close correlation between structural membrane deformability and their abnormal functions as a consequence of increased oxidative stress (Pieper et al. 1995; Karasu 1999). On the other hand, it was demonstrated previously that neither acute nor chronically diabetes affected superoxide dismutase (SOD) activities in different tissues (Koçak et al. 2000; Ozansoy et al. 2001; Zobali et al. 2002). The selective increase in GSHPx and CAT but not SOD in diabetic tissues has also been reported by other laboratories (Wohaieb and Godin 1987; Pieper et al. 1995). For this reason, in the present study we did not measure myocardial SOD activity. In spite of increased activity of GSHPx and CAT, the TBARS levels were still found to be higher in the diabetic hearts, suggesting that oxidative stress exceeds the capacity of antioxidant enzymes to scavenge oxygen free radicals (Kakkar et al. 1996).

Hyperlipidemia alone may be the reason for enhanced levels of TBARS in diabetics, since previous research has directly linked hyperlipidemia with increased serum and tissue concentrations of lipid peroxidation products (Kubow et al. 1996; Ozansoy et al. 2001). Simvastatin, an HMG-CoA reductase inhibitor, competitively inhibits HMG-CoA reductase and catalyzes the rate-limiting steps in cholesterol biosynthesis. The resultant reduction in hepatocyte cholesterol concentration triggers increased expression of hepatic low-density lipoprotein (LDL) receptors, which clear LDL and LDL precursors from the circulation. The metabolic and structural findings of the present study support the results of our previous study with

a different lipid-lowering drug, gemfibrozil (Ozansoy et al. 2001). Previously, it was shown that in diabetic rats simvastatin lowered 8-epi-prostaglandin- $F_2\alpha$ level, which is an indicator of oxidative stress (Palmer et al. 1998), and decreased the concentration of serum hydroperoxides in diabetic patients (Balabolkin et al. 1992). Similarly, cholesterol-lowering treatments with other statins or probucol effectively reduce oxidative modification of the lipoprotein fraction containing intermediate density lipoprotein (IDL) and LDL in hypercholesterolemic type 2 diabetic patients (Karasu 1998; Harada et al. 1999; El-Swefy et al. 2000).

We were first to show that simvastatin treatment positively affected GSHPx and CAT activities in the diabetic rat myocardium, which may be the consequence of its decreasing effects on hydroperoxides. The effects of other HMG-CoA reductase inhibitors on endogenous antioxidant enzyme activities have been investigated in diabetic or hypercholesterolemic animals, and fluvastatin or lovastatin were found to normalize antioxidant enzyme activities in those animals. The metabolic benefits of these drugs were associated with renal and hepatic improvements (Chen et al. 1997; Yamamoto et al. 1998; Kurusu et al. 2000). Antiangiopathic and antiatherogenic ultrastructural manifestations in the diabetic myocardium are in agreement with the results of other studies, demonstrating beneficial cardiovascular effects of some antioxidants in diabetic animals (Rösen et al. 1995; Fitzl et al. 1999; Koçak et al. 2000; Stefek et al. 2000; Ozansoy et al. 2001; Zobali et al. 2002).

Although it is widely accepted that the majority of clinical benefits obtained with statins is a direct result of their lipid-lowering properties, these agents appear to display additional cholesterol-independent effects on various aspects of cardiovascular disease, e.g. improving endothelial function, decreasing vascular inflammation and enhancing plaque stability (Chisolm et al. 1992; Vaughan et al. 1996). Some angiographic studies demonstrated that the beneficial effects observed with statins may be mediated by factors related to modulations of cell functions, such as migration and proliferation, thrombogenesis and stabilization of atherogenic plaques, presumably by inhibiting the biosynthetic pathway of isoprenoids from mevalonate (Maron et al. 2000).

In addition to the many different effects of simvastatin, our findings demonstrated that simvastatin acted also as an antioxidant in the diabetic rat myocardium. This was documented by suppressing lipid peroxidation induced by diabetes and also by normalizing cardiac GSHPx and CAT activities. Our results suggest that these properties of simvastatin may contribute to its antiangiopathic, antiatherogenic and cardioprotective effects.

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