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Effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on mitochondrial respiration in ischaemic dog hearts

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1 Effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, pravastatin and simvastatin, on the myocardial level of coenzyme Q_{10}, and on mitochrondrial respiration were examined in dogs.

2 Either vehicle (control), pravastatin (4 mg $kg^{-1}day^{-1}$), or simvastatin (2 mg $kg^{-1}day^{-1}$) was administered orally for 3 weeks. First, the myocardial tissue level of coenzyme Q_{10} was determined in the 3 groups. Second, ischaemia was induced by ligating the left anterior descending coronary artery (LAD) in anaesthetized open chest dogs, pretreated with the inhibitors. After 30 min of ischaemia, nonischaemic and ischaemic myocardium were removed from the left circumflex and LAD regions, respectively, and immediately used for isolation of mitochondria. The mitochondrial respiration was determined by polarography, with glutamate and succinate used as substrates.

3 Simvastatin significantly decreased the myocardial level of coenzyme Q_{10}, but pravastatin did not.

4 Ischaemia decreased the mitochondrial respiratory control index (RCI) in both groups. Significant differences in RCI between nonischaemic and ischaemic myocardium were observed in the control and simvastatin-treated groups.

5 Only in the simvastatin-treated group did ischaemia significantly decrease the ADP/O ratio, determined with succinate.

6 The present results indicate that simvastatin but not pravastatin may cause worsening of the myocardial mitochondrial respiration during ischaemia, probably because of reduction of the myocardial coenzyme Q_{10} level.

Keywords: 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors; ischaemic heart; mitochondria

Introduction

We (Ichihara *et al.*, 1993) have reported in an earlier paper that pretreatment of dogs with simvastatin but not pravastatin enhances contractile dysfunction of the myocardium in association with ATP reduction after reperfusion subsequent to ischaemia. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a key enzyme not only in cholesterol biosynthesis but also in ubiquinone biosynthesis (Rodwell *et al.*, 1976). Activity of HMG-CoA reductase has been reported in myocardial cells (Koga *et al.*, 1990) as well as in other organ cells (Rodwell *et al.*, 1976). Since ubiquinones play an important role in the mitochondrial respiratory chain, inhibition of HMG-CoA reductase may influence the myocardial energy generating system, particularly under pathophysiological conditions. Simvastatin may cause cardiac dysfunction during reperfusion after ischaemia through the inhibition of ubiquinone biosynthesis. Folkers and colleagues (1990) have demonstrated that lovastatin, an HMG-CoA reductase inhibitor, causes cardiac dysfunction associated with a decreased level of cardiac or serum ubiquinone in dogs and human patients. Because simvastatin and lovastatin are lipid-soluble HMG-CoA reductase inhibitors (Koga *et al.*, 1990), these drugs can enter the myocardial cell where they inhibit HMG-CoA reductase. These inhibitors reduce sterol synthesis in the heart to about 50% of the initial level (Koga *et al.*, 1990). Pravastatin, however, is water-soluble and cannot enter the myocardial cells. The present study, therefore, was undertaken to examine the influence of pravastatin and simvastatin on the myocardial ubiquinone levels and respiratory function of mitochondria isolated from dog hearts which had been made ischaemic.

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Methods

Preparation of animals

Beagles (NRD Beagle, Hokkaido, Japan) of either sex weighing 11.4 ± 0.6 kg were randomly assigned to either vehicle-treated (control), pravastatin-treated, or simvastatin-treated group. Pravastatin and simvastatin were given in a gelatin capsule. Pravastatin (4 mg $kg^{-1}day^{-1}$) and simvastatin (2 mg $kg^{-1}day^{-1}$) were administered orally to dogs once a day at 08 h 00 min for 3 weeks. An empty capsule was given to the control animals. All animals had free access to standard dog chow and water and were deprived of food for approximately 15 h before the experiments started. After 3 weeks of treatment, animals were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.), the trachea was intubated and ventilated with room air from a respirator. A left thoracotomy was performed between the fourth and fifth ribs to expose the left ventricular wall.

Assay of coenzyme Q_{10}

The left ventricular free wall was removed and frozen with liquid N_2, and then stored in liquid N_2 until assayed. After weighing, the frozen tissue was thawed and homogenized with 10 volumes of H_2O. The homogenate (0.25 ml) was incubated at 37°C with 0.1 ml of 4% sodium taurocholic acid-0.1 M Tris-HCl buffer (pH 8.0) and 0.2 ml of 2% pancreatin-0.1 M Tris-HCl (pH 8.0). After 30 min of incubation, 3.5 ml of ethanol, internal standard (2,3,6-trimethyl-5-decaprenyl-1,4-benzoquinone), and 2% ferric chloride were added and ubiquinone was extracted with 5 ml of hexane for 5 min. Then the mixture was centrifuged and the supernatant was dried. The residue was dissolved in 0.2 ml of isopropylalcohol and an aliquot of the extract was injected into high performance liquid chromatography (Shimazu LC-6A, Kyoto, Japan). The peak was quantified by u.v. detection (Shimazu SPD-6A, Kyoto, Japan).

Ischaemic experiments

In this series of experiments, the main trunk of the left anterior descending coronary artery (LAD) was dissected free from the adjacent tissue and was then loosely encircled with a silk thread for ligation. Ischaemia was produced by tightening this thread. Aortic blood pressures were measured via a cannula introduced from the left femoral artery near the aortic arch. Heart rate was counted from the QRS signals from an ECG taken from the standard limb lead II. After control observations were completed, the ligature around the LAD was tied for 30 min in each animal. Samples of non-ischaemic and ischaemic myocardium were taken from the left circumflex coronary artery (LCX) region and LAD region, respectively.

Isolation of mitochondria

The myocardial LAD and LCX samples were immediately placed in cold isolation buffer consisting of KCl 180 mM, EDTA-2K 10 mM, Tris 10 mM and 0.5% bovine serum albumin (BSA) (pH 7.4). Basically, the procedure of Vercesi *et al.* (1978) was used for isolation of cardiac muscle mitochondria. The tissue was finely minced and suspended in 10 volumes of isolation buffer containing nagarse 0.1 mg ml^{-1}. After 15 min of incubation at 0°C with occasional stirring, the medium was discarded. The minced myocardium was washed once with the isolation buffer to remove excess nagarse. The tissue was resuspended in 10 volumes of buffer and homogenized with a glass homogenizer and a teflon pestle. The homogenate was centrifuged at 600 *g* for 5 min to obtain the supernatant. The supernatant was then centrifuged at 10,000 *g* for 10 min, and the pellet obtained was washed once. The final mitochondrial pellet was suspended in isolation buffer and used immediately for experiments on mitochondrial respiration. Protein concentration was determined by the method of Lowry *et al.* (1951) with BSA (Fraction V) used as a standard. The yield of the mitochondria in the LAD region was 5.19 ± 0.20 mg protein g^{-1} tissue ($n=20$), and in the LCX region was 5.33 ± 0.26 mg protein g^{-1} tissue ($n=20$). There was no significant difference between groups.

Assay of mitochrondrial respiration

Mitochondrial respiration was measured polarographically at 25°C with an oxygen monitor (OHB-100, Otsuka Electronics Co., Ltd., Osaka, Japan) equipped with an oxygen electrode modified by Hagihara (1961). Approximately 0.1 – 1 mg mitochondrial protein, 250 nmol of ADP, either 10 mM glutamate or 10 mM succinate with rotenone (8 mg ml^{-1}) as a substrate, and assay medium containing 3 mM K_2HPO_4, 1 mM EDTA-2K, 10 mM Tris and 250 mM sucrose (pH 7.4) were used for the assay. Mitochondrial protein was added to a cuvette containing assay medium equilibrated with air at 25°C, and then respiratory substrate was added. After 2 min-stabilization, state 3 respiration was initiated by addition of ADP. Designations (QO_3 for ADP-stimulated and QO_4 for ADP-limited respiration) and calculation of the respiratory control index (RCI) and ADP/O ratios were carried out according to the method described by Chance *et al.* (1955).

Statistical analysis

All values are means ± s.e.mean. The significance of differences between groups was evaluated by one-way analysis of variance followed by the unpaired *t* test with the Bonferroni procedure. A *P* value less than 0.05 was considered statistically significant.

Results

Myocardial coenzyme Q_{10} level

We first established the effects of pravastatin and simvastatin on the myocardial coenzyme Q_{10} level (Figure 1). The myocardial tissue level of coenzyme Q_{10} in the simvastatin-treated animals was significantly lower than those in the control and pravastatin-treated animals. There was no significant difference in the level of coenzyme Q_{10} between control and pravastatin-treated animals.

Haemodynamics

Blood pressure and heart rate before LAD ligation were not modified by pravastatin and simvastatin treatments (Figure 2). The LAD ligatation appeared to decrease systolic blood pressure in all 3 groups and decreased diastolic pressure in pravastatin-treated group. Heart rate was not changed appreciably by the LAD ligation.

Serum cholesterol level

The levels of serum cholesterol in the control and simvastatin-treated animals were not significantly changed by 3 weeks of administration (Figure 3). However, administration of pravastatin for 3 weeks significantly decreased the levels of serum cholesterol.

RCI

RCI values were calculated by dividing QO_3 by QO_4 (Figure 4). When glutamate was used as a substrate, ischaemia significantly decreased RCI in the control and simvastatin-treated animals, because QO_3 decreased and QO_4 increased during ischaemia. In the pravastatin-treated dogs, RCI tended to be decreased by ischaemia, but the difference was not statistically significant. When succinate was used as a substrate, RCI was not changed by ischaemia either in the control or pravastatin-treated animals. However, in the simvastatin-treated animals, RCI decreased significantly during ischaemia.

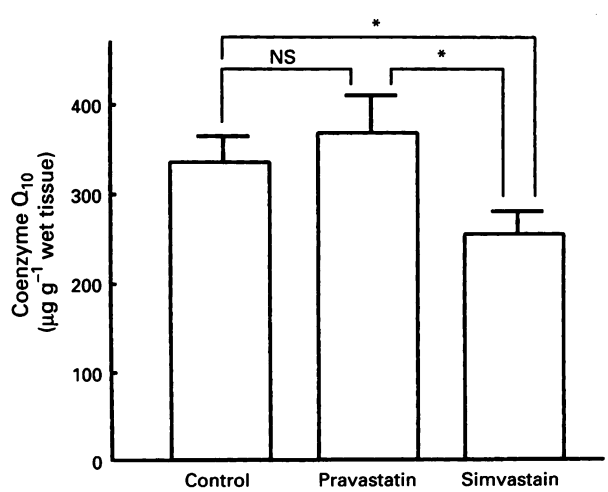

Figure 1 Myocardial level of coenzyme Q_{10} in the pravastatin- and simvastatin-treated dogs. Either an empty geratin capsule, or a capsule filled with pravastatin (4 mg kg^{-1}) or simvastatin (2 mg kg^{-1}) were given orally once a day to the dogs for 3 weeks. After administration, the left ventricular free wall was removed under pentobarbitone-anaesthesia. Values are means ± s.e.mean of 6 observations in each group.
*$P<0.05$, compared with either control or pravastatin-treated dogs.

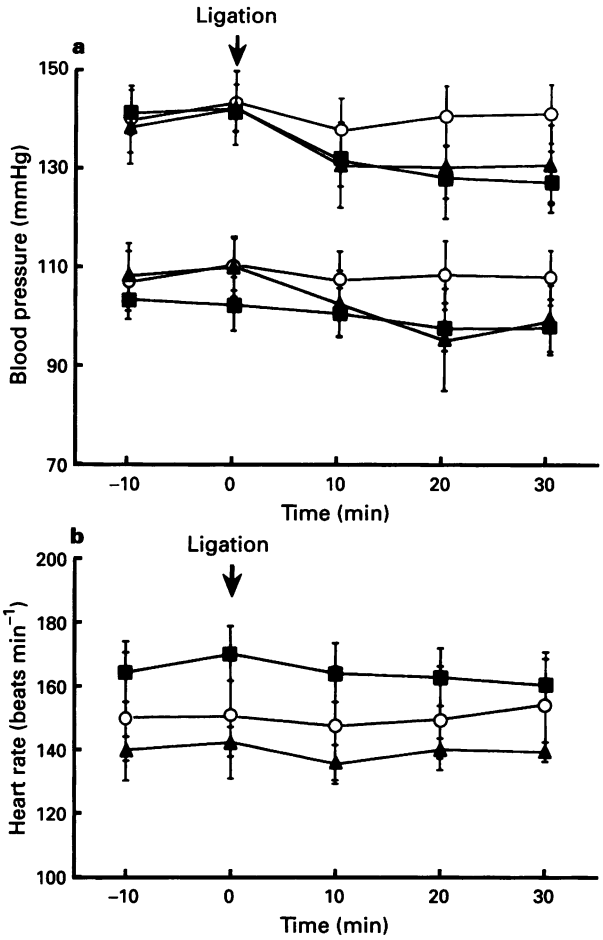

Figure 2 Haemodynamic changes: systolic (upper lines in a) and diastolic pressure (lower lines in a), and heart rate (b) were measured. Pravastatin (▲) and simvastatin (■) were given orally as in Figure 1. In the control group (○), dogs were given an empty capsule. After 3 weeks, the left anterior descending coronary artery (LAD) was ligated for 30 min under pentobarbitone-anaesthesia. All values are mean ± s.e.mean (6 observations in control, and 7 observations in the other groups).

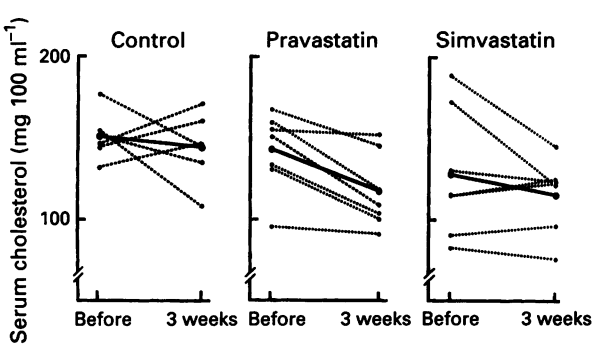

Figure 3 Serum cholesterol levels before and 3 weeks after administration of either pravastain or simvastatin. Individual observations (dotted lines); mean value (solid line).
$^{**}P<0.01$, compared with the value obtained before administration.

ADP/O ratio

The ADP/O ratio indicates ATP production per unit of oxygen (Figure 5). There was no significant difference in the non-ischaemic ADP/O ratio between the 3 groups. When glutamate was used as a substrate, the ADP/O ratio in all 3 groups was not altered by ischaemia. When succinate was used as a substrate, the ADP/O ratio in the simvastatin-treated hearts was significantly decreased by ischaemia. However, the ADP/O ratio in the control and pravastatin-treated animals was unchanged during ischaemia.

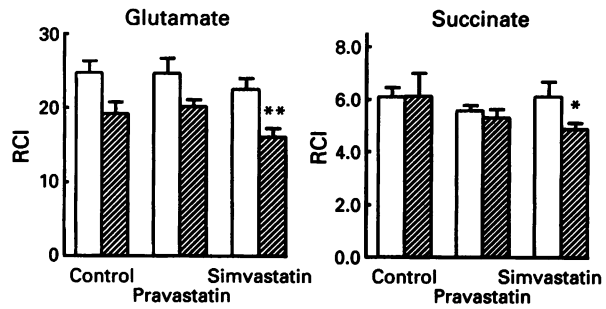

Figure 4 Effects of pravastatin and simvastatin on respiratory control index (RCI) in dog heart mitochondria. To make the heart ischaemic, the left anterior descending coronary artery (LAD) was ligated for 30 min. The LAD region and circumflex region were taken as ischaemic (hatched column) and non-ischaemic (open column) myocardial samples, respectively. Mitochondria were isolated by differential centrifugation and immediately used for assay of respiratory function using an oxygen monitor. RCI is the ratio of state 3 respiration to state 4 respiration.
$^{*}P<0.05$; $^{**}P<0.01$, compared with non-ischaemia in each group.

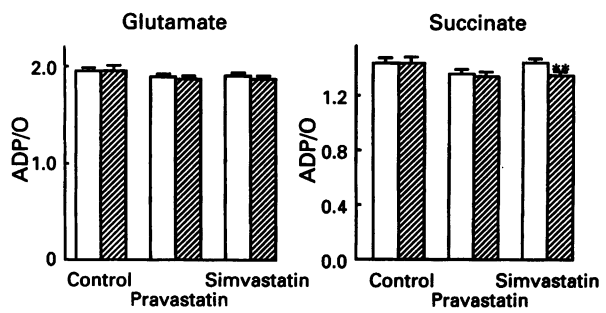

Figure 5 Effects of pravastatin and simvastatin on ADP/O ratio in dog heart mitochondria. Experimental protocol and symbols are the same as those in Figure 4. ADP/O ratio is the ability to produce ATP per unit of oxygen.
$^{**}P<0.01$, compared with non-ischaemia in each group.

Discussion

HMG-CoA reductase inhibitors prevent ubiquinone biosynthesis as well as cholesterol biosynthesis (Rodwell *et al.*, 1976), because mevalonate which is a product of HMG-CoA reductase reaction is a precursor in both cholesterol and ubiquinone biosynthesis. Ubiquinone is essential for the production of energy through oxidative phosphorylation in mitochondria. Mitochrondrial respiration produces energy for myocardial contraction. Inhibition of ubiquinone biosynthesis may impair the mitochondrial energy generating system, leading to cardiac dysfunction. In fact, we (Ichihara *et al.*, 1993) have demonstrated that simvastatin but not pravastatin worsens the recovery of myocardial contractile function during reperfusion following brief ischaemia. Because simvastatin is lipid-soluble, whereas pravastatin is water-soluble (Koga *et al.*, 1990), only simvastatin may enter the myocardial cell and prevent ubiquinone synthesis through inhibition of the HMG-CoA reductase. In the present study, simvastatin significantly decreased the myocardial level of ubiquinone (coenzyme Q_{10}). However, administration of pravastatin and simvastatin for 3 weeks did not alter the mitochondrial respiratory indices in the nonischaemic heart. On the other hand,

some mitochondrial respiratory indices in the ischaemic heart showed that simvastatin worsened the impairment of mitochondrial respiratory function caused by ischaemia. Values of RCI measured with glutamate and succinate, and the ADP/O ratio measured with succinate in the simvastatin-treated ischaemic heart were significantly lower than those in the nonischaemic heart (Figures 4 and 5). Values of RCI and the ADP/O ratio in the pravastatin-treated heart appeared to be decreased by ischaemia, but the difference between non-ischaemic and ischaemic hearts was not statistically significant. These results may indicate that simvastatin enhances the ischaemic influence on mitochondrial respiration and ATP generation in the myocardium.

Effects of simvastatin on the ADP/O ratio were clearly observed when succinate was used as a substrate (Figure 5). This result indicates that worsening of mitochondrial respiration with simvastatin may be due to reduction of ubiquinone levels. Electrons generated from glutamate oxidation flow into the respiratory chain at the NAD-related enzyme step, whereas those from succinate oxidation enter at the ubiquinone step. When a HMG-CoA reductase inhibitor attenuates the electron flow rate through the ubiquinone step to 70%, for example, ATP generation decreases stoichiometrically from 2 to 1.4 mol per mol succinate (70% of initial), whereas it decreases from 3 to 2.4 mol per mol glutamate (80% of initial). Decrease in the ubiquinone level should reduce the ADP/O ratio determined with succinate more than that with glutamate (Satoh *et al.*, 1994).

The effects of HMG-CoA reductase inhibitors on respiratory indices obtained in the present study were so weak, because we used rather low dosages of the inhibitors (4 mg $kg^{-1}day^{-1}$ of pravastatin and 2 mg $kg^{-1}day^{-1}$ of simvastatin). Doses for oral administration in other papers are 5–20 mg $kg^{-1}day^{-1}$ for mice (Koga *et al.*, 1990), 25 mg $kg^{-1}day^{-1}$ for rats (Germershausen *et al.*, 1989), and 50 mg $kg^{-1}day^{-1}$ for rabbits and hamsters (Watanabe *et al.*, 1988; Bélichard *et al.*, 1993). Because the clinical dose of HMG-CoA reductase inhibitors is about 0.3–1 mg $kg^{-1}day^{-1}$, and because the inhibitory effect of simvastatin on cholesterol synthesis is twice as potent as that of pravastatin (Tsujita *et al.*, 1986), we chose the doses used in the present study. However, pravastatin decreased serum cholesterol level more than simvastatin (Figure 3).

Tissue selectivities among HMG-CoA reductase inhibitors are pointed out by many investigators (Koga *et al.*, 1990; Parker *et al.*, 1990). Pravastatin is a relatively weak inhibitor of cholesterol synthesis in extrahepatic cells as compared with other HMG-CoA reductase inhibitors but is very effective in inhibiting cholesterol synthesis in hepatocytes (Tsujita *et al.*, 1986; Parker *et al.*, 1990; Koga *et al.*, 1990). On the other hand, simvastatin inhibits HMG-CoA reductase in any organ (Koga *et al.*, 1990; Parker *et al.*, 1990). Microautoradiography using ^{14}C-labelled drugs shows that simvastatin is taken up in the cells of both liver and spleen, whereas pravastatin is taken up only in liver cells (Koga *et al.*, 1992). Because simvastatin is lipid-soluble, it can penetrate the cell membrane and enter the cells in any organ. Pravastatin is water-soluble, and cannot enter the cell except for liver cells in which a specific uptake mechanism exists (Yamazaki *et al.*, 1993). We have demonstrated in the rat that simvastatin worsens the ischaemia-induced damage of mitochondrial respiration in the heart (Satoh & Ichihara, 1995), and that both simvastatin and pravastatin enhance the ischaemia-induced worsening of mitochondrial respiration in the liver (Satoh *et al.*, 1994). In the present study, ischaemia-induced impairment of mitochondrial respiration in simvastatin-treated hearts was greater than that in the pravastatin-treated hearts. Simvastatin may easily enter the myocardial cells and inhibit HMG-CoA reductase as a result of which energy production deteriorates through reduction of the ubiquinone level, particularly in the ischaemic heart. This may cause myocardial dysfunction after reperfusion following ischaemia. We (Ichihara *et al.*, 1993) have predicted in our previous paper that the lipid-soluble inhibitors may aggravate ischaemic heart disease or coronary artery disease. Recently, clinical studies on long-term treatment with HMG-CoA reductase inhibitors have been successively reported (Blankenhorn *et al.*, 1993; Waters *et al.*, 1993; Furberg *et al.*, 1994; Pedersen *et al.*, 1994; Oliver *et al.*, 1994). In all reports, HMG-CoA reductase inhibitors slow and reduce atherosclerosis in the coronary arteries. However, only pravastatin reduces cardiac death and cardiac event rates (Furberg *et al.*, 1994). Lovastatin reduces atherosclerosis but does not reduce the cardiac event rate (Blankenhorn *et al.*, 1993; Waters *et al.*, 1993). Simvastatin reduces both atherosclerosis and cardiac events only in patients with high cholesterol (mean 6.74 mmol l^{-1}) (Pedersen *et al.*, 1994). It does not affect cardiac death and cardiac event rate in patients with a medium cholesterol level (mean 6.35 mmol l^{-1}) (Oliver *et al.*, 1994). These clinical data suggest that the beneficial effect of lovastatin and simvastatin in coronary artery disease due to regression of atherosclerosis is attenuated by the deleterious effect of the inhibitors on the mitochondrial energy generating system in the myocardium.

In conclusion, a HMG-CoA reductase inhibitor, particularly a lipid-soluble one, may result in worsening of the mitochondrial respiration during ischaemia, and may cause cardiac dysfunction.

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