Limitation of heart growth in neonatal piglets by simvastatin and atorvastatin: comparison with pravastatin

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Satoh, Kumi, Fumiyo Shirota, Takahiko Tsunajima, Cathy J. Beinlich, Howard E. Morgan, and Kazuo Ichihara. Limitation of heart growth in neonatal piglets by simvastatin and atorvastatin: comparison with pravastatin. Am J Physiol Heart Circ Physiol 280: H2746–H2751, 2001.—The pig heart grows rapidly in the first few days after birth. We examined the effects of simvastatin, atorvastatin, and pravastatin on heart growth in piglets. After vehicle, 2 mg·kg−1·day−1 simvastatin, 2 mg·kg−1·day−1 atorvastatin, or 4 mg·kg−1·day−1 pravastatin were administered orally for 6 days, the thoracic cavity was opened, and the heart was removed under pentobarbital sodium (30 mg/kg ip) anesthesia. The heart was perfused to remove residual blood. After the heart was blotted dry, the right and left ventricular free walls were dissected. Each free wall was weighed and used for determination of DNA, RNA, and protein concentrations with mitogen-activated protein (MAP) kinase activity. Simvastatin and atorvastatin resulted in smaller increases with age in the weight, concentrations of RNA and protein, and activity of MAP kinase in the left ventricular free wall, whereas pravastatin did not. The parameters of heart growth in the right ventricular free wall were not appreciably affected by either drug. The blood pressure and heart rate were not changed by the treatments. These results suggest that simvastatin and atorvastatin interfere with heart growth in neonatal piglets after birth, especially in the left ventricular free wall.

METHODS

This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, Revised 1985).

Animals. Neonatal piglets were obtained within 2 h after birth from a local commercial breeder. Five piglets obtained within 2 h after birth and five piglets obtained 6 days after birth. We examined the effects of simvastatin, atorvastatin, and pravastatin on heart growth in piglets. After vehicle, 2 mg·kg−1·day−1 simvastatin, 2 mg·kg−1·day−1 atorvastatin, or 4 mg·kg−1·day−1 pravastatin were administered orally for 6 days, the thoracic cavity was opened, and the heart was removed under pentobarbital sodium (30 mg/kg ip) anesthesia. The heart was perfused to remove residual blood. After the heart was blotted dry, the right and left ventricular free walls were dissected. Each free wall was weighed and used for determination of DNA, RNA, and protein concentrations with mitogen-activated protein (MAP) kinase activity. Simvastatin and atorvastatin resulted in smaller increases with age in the weight, concentrations of RNA and protein, and activity of MAP kinase in the left ventricular free wall, whereas pravastatin did not. The parameters of heart growth in the right ventricular free wall were not appreciably affected by either drug. The blood pressure and heart rate were not changed by the treatments. These results suggest that simvastatin and atorvastatin interfere with heart growth in neonatal piglets after birth, especially in the left ventricular free wall.

mitogen-activated protein kinase

3-HYDROXY-3-METHYLGLUTARYL (HMG)-CoA reductase inhibitors are widely used for treatment of patients with hypercholesterolemia and hyperlipidemia. Clinical studies (5, 11, 21, 22, 24, 31, 33) have demonstrated that the beneficial effects of HMG-CoA reductase inhibitors on coronary heart diseases are linked to their hypocholesterolemic properties. Several recent studies, however, have reported that some beneficial effects of HMG-CoA reductase inhibitors, such as regression of atherosclerosis (8), inhibition of tumor cell growth (17), and reduction of cardiac hypertrophy (20), are independent of their hypocholesterolemic properties. For example, HMG-CoA reductase inhibitors exert a direct antiatherosclerotic effect on the arterial wall through inhibition of vascular smooth muscle cell migration and proliferation (4).

The HMG-CoA reductase that converts HMG-CoA to mevalonic acid is the rate-limiting enzyme of cholesterol biosynthesis. Mevalonic acid is also the precursor of isoprenoid groups that are incorporated into many important products, including heme A, dolichol, ubiquinones, isopentenyl adenine (present in some tRNAs), and farnesylated proteins, as well as cholesterol (23). Isoprenoids are required for isoprenylation of several proteins involved in signal transduction, including small-molecular-weight GTP-binding proteins and the γ-subunits of heterotrimeric GTP-binding proteins (12). These proteins play an important role in many intracellular signal transduction systems, including those concerned with cell proliferation, apoptosis, maintenance of cellular functions, and cell growth.

The newborn pig heart is a convenient model to study physiological cardiac growth. The pig heart grows at a maximal rate in the first few days of life due to both volume and pressure overloads imposed on the heart at birth (2, 3). During this period, the left ventricle grows rapidly, and the right ventricle grows slowly (7). The present study was designed to examine whether simvastatin and atorvastatin, lipophilic HMG-CoA reductase inhibitors, could modify heart growth in neonatal piglets as one of the cellular functions regulated by the signal transduction system and to compare the heart growth effects with those of pravastatin, a hydrophilic HMG-CoA reductase inhibitor.

antihyperlipidemic effect on the arterial wall through inhibition of vascular smooth muscle cell migration and proliferation (4).
birth were used for determination of body weight and right ventricular free wall (RVFW) and left ventricular free wall (LVFW) weights. In the pharmacologically treated groups, piglets were orally given either 0.5% carboxymethylcellulose as vehicle (20 piglets), 2 mg·kg⁻¹·day⁻¹ simvastatin (10 piglets), 2 mg·kg⁻¹·day⁻¹ atorvastatin (10 piglets), or 4 mg·kg⁻¹·day⁻¹ pravastatin for 6 days (10 piglets). Piglets at the age of 5 days old (6 days after birth) were delivered to our laboratory, heparinized with heparin sodium (40 mg/kg ip), and anesthetized with pentobarbital sodium (30 mg/kg ip). Blood samples were taken from the chest cavity when the heart was excised. Immediately after excision, the heart was rinsed with Krebs-Henseleit bicarbonate buffer aerated with 95% O₂-5% CO₂ and perfused by the Langendorff technique with the same buffer for ~1 min to remove residual blood in the coronary vessels. After the heart was blotted dry, the atria, great vessels, and fibrous, valvular, and fatty tissues were removed, and the RVFW and LVFW were weighed, frozen in liquid nitrogen, and then stored at ~80°C.

**RNA, DNA, and protein assays.** The frozen myocardium was pulverized with a mortar and pestle in liquid nitrogen. A small part of frozen tissue powder was weighed before and after drying in an oven overnight to determine the tissue water content. Another part of the tissue powder was weighed and put into a cold-tared Corex tube containing 6% perchloric acid. After extraction with a vortex using a Teflon pestle, the pellet obtained by centrifugation was rinsed several times and used for determination of RNA, DNA, and protein concentrations. RNA analysis was performed according to the method of Munro and Fleck (19). DNA concentration was determined from the absorbance at 268 and 284 nm by the method of Tsanev and Markov (32). Concentrations of RNA and DNA were expressed in milligrams per gram of dry weight, and total content of RNA and DNA was expressed in milligrams per heart portion. Protein concentration was determined by the method of Lowry (16) using bovine serum albumin (fraction V, Sigma; St. Louis, MO) as the standard.

**Determination of mitogen-activated protein kinase activity.** The remainder of the frozen tissue powder was weighed and homogenized in tissue weight (g) × 10 ml of 10 mM Tris·HCl (pH 7.4) containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The homogenate was centrifuged at 1,000 × g for 10 min, and the resultant supernatant was again centrifuged at 105,000 × g for 100 min. The supernatant obtained was used as the cytosolic fraction for the assay of mitogen-activated protein (MAP) kinase. The activity of MAP kinase was determined by using a p42/p44 MAP kinase enzyme assay system (Amer- sham; Buckinghamshire, UK). Briefly, p42/44 MAP Kinase catalyzes transfer of the γ-phosphate group of ATP to a peptide that is highly selective for p42/44 MAP kinase. The reaction was initiated by the addition of magnesium [γ-³²P]ATP. After 30 min of incubation at 30°C, a binding paper disc to which the peptide was bound was washed from the unincorporated radioactivity and counted with a scintillation counter for ³²P.

**Determination of blood pressure and heart rate.** Another 36 piglets were prepared for determination of blood pressures and heart rates. The piglets were used within 2 h after birth (6 piglets) or 6 days after oral administration of vehicle (7 piglets), 2 mg·kg⁻¹·day⁻¹ simvastatin (7 piglets), 2 mg·kg⁻¹·day⁻¹ atorvastatin (7 piglets), and 4 mg·kg⁻¹·day⁻¹ pravastatin (9 piglets). Within 2 h or 6 days after birth, piglets were anesthetized with pentobarbital sodium (30 mg/kg ip), and the arterial systolic and diastolic blood pressures were measured via a cannula inserted into the left carotid artery. Heart rate was counted from electrocardiogram limb lead II.

**Statistical analysis.** Values are expressed as means ± SE. The significance of differences between groups was evaluated by one-way analysis of variance, followed by the Tukey-Kramer multiple test. *P < 0.05 was considered statistically significant.

### RESULTS

**Heart growth after 6 days.** Table 1 shows the body weight and the weight of the RVFW and LVFW of the myocardium in 0-day-old and 5-day-old piglets. Six days after birth (5 days old), the body weight significantly increased by ~50%. The LVFW weight significantly increased for 6 days. The LVFW of 5-day-old piglets became 2.4 times larger than that of the 0-day-old piglets. However, the increase in the RVFW weight for 6 days was not statistically significant.

**Serum cholesterol levels.** Administration of 2 mg·kg⁻¹·day⁻¹ simvastatin, 2 mg·kg⁻¹·day⁻¹ atorvastatin, or 4 mg·kg⁻¹·day⁻¹ pravastatin for 6 days significantly lowered the serum cholesterol level compared with vehicle (133 ± 6 mg/100 ml in the vehicle-treated group, 78 ± 16 mg/100 ml in the simvastatin-treated group, 109 ± 8 mg/100 ml in the atorvastatin-treated group, and 84 ± 8 mg/100 ml in the pravastatin-treated group).

**Heart weights.** The ratios of the weights of RVFW and LVFW to body weight in the pharmacologically treated groups are shown in Fig. 1. Mean body weights were 1.91 ± 0.06, 2.00 ± 0.13, 1.97 ± 0.16, and 1.85 ± 0.11 kg in the vehicle-, simvastatin-, atorvastatin-, and pravastatin-treated groups, respectively. There were no significant differences in body weights between them. The ratios of the LVFW to the body weights in the simvastatin- and atorvastatin-treated groups were significantly lower than those in the vehicle- and pravastatin-treated groups. However, the RVFW weight-to-body weight ratio was not altered by either treatment.

**RNA, DNA, and protein concentrations.** The concentrations of RNA, DNA, and protein in the LVFW in each treated group are summarized in Table 2. In all groups, the RNA concentration in the LVFW was significantly higher than that in the RVFW. The protein concentration in the LVFW was also higher than that in the RVFW in the vehicle- and pravastatin-treated groups.

<table>
<thead>
<tr>
<th>Table 1. Heart growth of neonatal piglets for 6 days after birth</th>
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<tbody>
<tr>
<td>Heart Growth</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Body weight, kg</td>
</tr>
<tr>
<td>RVFW weight, g</td>
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<tr>
<td>LVFW weight, g</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of observations. Hearts were removed within 2 h after birth (n = 5) or 6 days after birth (5 days old; n = 5). RVFW and LVFW, right and left ventricular free wall, respectively; %Change, percent increment in each parameter for 6 days. *P < 0.05 compared with piglet < 2 h old.
groups. On the other hand, in the simvastatin- and atorvastatin-treated groups, the protein concentration in the LVFW was lower than that in the RVFW. The DNA concentration was not different between the RVFW and LVFW in either treated group. In the simvastatin-treated group, the concentrations of RNA and protein in the LVFW were significantly lower than those in the vehicle-treated group. Total RNA, DNA, and protein in the LVFW were significantly higher than those in the RVFW because of the large mass of the LVFW (Fig. 1 and Table 2). Pravastatin did not change the total amount of RNA, DNA, and protein in both the RVFW and LVFW. However, the total amounts of RNA, DNA, and protein in the LVFW in the simvastatin- and atorvastatin-treated groups were significantly lower than those in the vehicle-treated group. Total RNA, DNA, and protein in the RVFW were not modified by drug treatments.

**MAP kinase activity.** The activities of MAP kinase in the RVFW and LVFW in the vehicle-treated group were 17.5±2.0 and 21.4±1.5 pmol Pi·min⁻¹·mg protein⁻¹, respectively. The enzyme activities in the drug-treated groups were normalized to those in the vehicle-treated group and are shown in Fig. 2. Pravastatin did not affect MAP kinase activity in both the RVFW and LVFW. However, simvastatin and atorvastatin significantly decreased MAP kinase activity in the LVFW. MAP kinase activity in the RVFW was not significantly altered by either treatment.

**Tissue water contents.** The tissue water contents in the frozen sample used above in the vehicle-treated group were 0.839±0.003 (RVFW) and 0.843±0.003 (LVFW) water wt/tissue wet wt. There was no significant difference in the ratios of water weight to wet

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**Table 2. Effects of simvastatin, atorvastatin, and pravastatin on RNA, DNA, and protein concentrations in the LVFW of neonatal piglets**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Simvastatin</th>
<th>Atorvastatin</th>
<th>Pravastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>14.8±0.3</td>
<td>13.1±0.4††</td>
<td>13.7±0.5††</td>
<td>15.3±0.4</td>
</tr>
<tr>
<td>DNA</td>
<td>12.8±0.3</td>
<td>12.7±0.6</td>
<td>12.4±0.5†</td>
<td>13.8±0.5</td>
</tr>
<tr>
<td>Protein</td>
<td>846.1±8.6</td>
<td>721.8±12.2††</td>
<td>750.1±43.4††</td>
<td>873.0±7.8</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>1.15±0.04</td>
<td>1.05±0.06</td>
<td>1.13±0.08</td>
<td>1.12±0.06</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>66.0±1.0</td>
<td>58.0±2.9§</td>
<td>60.4±2.6§</td>
<td>64.1±2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of observations. Vehicle (0.5% carboxymethylcellulose, n = 20), 2 mg·kg⁻¹·day⁻¹ simvastatin (n = 10), 2 mg·kg⁻¹·day⁻¹ atorvastatin (n = 10), or 4 mg·kg⁻¹·day⁻¹ pravastatin (n = 10) was orally administered for 6 days, starting just after birth. Experimental protocols were the same as those in Fig. 1. After they were weighed, the RVFW and LVFW were frozen in liquid nitrogen and stored at −80°C until each assay was done. The concentrations of RNA, DNA, and protein in the LVFW are expressed as milligrams per gram dry weight. *P < 0.05 compared with the vehicle-treated group; †P < 0.05 compared with the pravastatin-treated group.

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**Fig. 2.** The effect of simvastatin, atorvastatin, and pravastatin on mitogen-activated protein (MAP) kinase-specific activity in the RVFW and LVFW. The experimental protocol and symbols are the same as those in Fig. 1. *P < 0.05 compared with the vehicle-treated group; †P < 0.05 compared with the pravastatin-treated group.
tissue weight between the vehicle-treated and the drug-treated groups.

**Blood pressures and heart rates.** The systolic and diastolic blood pressures and heart rates of the piglets measured within 2 h after birth and those measured 6 days after drug treatments are summarized in Table 3. The systolic and diastolic blood pressures significantly increased during 6 days after birth. Although the systolic pressures in all drug-treated groups appeared to be low compared with that in the vehicle-treated group, there were no significant differences among them. The heart rate in the simvastatin-treated group was significantly increased with age, but the value was not different from the vehicle-treated group 6 days after birth. The heart rates in the other groups were not significantly changed with age and by drug treatments.

**DISCUSSION**

The LVFW weight 6 days after birth was 2.4 times the weight just after birth (Table 1). During this 6-day period, the body weight increased 1.5 times. Because both volume and pressure loads are imposed on the left ventricle at birth, the LVFW cell growth occurs at the maximum rate (2, 3). The present study was undertaken in whole heart tissue, which is of heterogeneous cell composition. The myocardium contains nonmyocytes, such as vascular smooth muscle and endothelial cells and fibroblasts, as well as myocytes. However, 90% of postnatal growth can be accounted for by hypertrophy of existing cardiac myocytes in the neonatal pig heart (35). The rapid growth of the LVFW during the early days of life was accompanied by a rapid increase in myocyte size (2). Therefore, growth of the neonatal piglet heart in the present study may be mainly due to growth of the cardiac myocyte.

We (13, 27) previously demonstrated that HMG-CoA reductase inhibitors having a lipophilic property worsen the postischemic contractile dysfunction in the reperfused heart after a brief period of ischemia. Because a HMG-CoA reductase inhibitor prevents mevalonic acid formation, which is a precursor of several substances including coenzyme Q10 and isoprenoids, the mechanisms underlying this phenomenon are probably 1) inhibition of coenzyme Q10 biosynthesis in the myocardium, leading to dysfunction of mitochondrial energy-generating system (28); and 2) reduction of isoprenylation of the γ-subunits of heterotrimeric GTP-binding proteins, leading to a decrease in cardiac contractile response to catecholamines (12). When the myocardial contraction decreases under some pathophysiological conditions, such as ischemia-reperfusion, catecholamines are released and restore the myocardial contraction (26). A lipophilic HMG-CoA reductase inhibitor may enter the cardiac myocyte (14), prevent isoprenoid formation, disturb the intracellular signal transduction of catecholamines, and lead to cardiac contractile dysfunction (10).

When a lipophilic HMG-CoA reductase inhibitor prevents isoprenylation of several functional proteins in the cell, several cell functions may be influenced. In the present study, simvastatin and atorvastatin attenuated the cardiac myocyte growth in the LVFW of neonatal piglets that should occur within 6 days after birth (Fig. 1). Because the rapid growth of the LVFW is primarily caused by enlargement of cardiac myocytes, but not by an increase in the cell number (2), simvastatin and atorvastatin may not prevent myocyte proliferation but myocyte growth. In fact, the DNA contents that may be derived from cardiac myocyte nuclei were not changed by simvastatin and atorvastatin treatments (Fig. 2). However, the total amount of DNA was decreased by simvastatin and atorvastatin, because both drugs inhibited the weight gain of the LVFW. Simvastatin and atorvastatin significantly decreased the ratio of protein to DNA, indicating a decrease in cell size (Table 2). This finding also suggests that these drugs limited the rate of normal cardiac myocyte growth in the LVFW during the neonatal period by restraining hypertrophy or cell size.

The rapid growth of the left ventricle of the neonatal piglet was paralleled by an increase in RNA content that was greater in the left ventricle than the right ventricle (6). Simvastatin and atorvastatin reduced the contents of RNA and protein in the LVFW in association with decreased activities of MAP kinase (Table 2 and Fig. 2). Many factors, such as ventricular stretch (25), catecholamines (7), and several hormones including angiotensin II (1), regulate cardiac growth through the intracellular signal transduction (18). MAP kinase is one of a family of serine/threonine protein kinases activated by various stimuli involved in cardiac growth, such as angiotensin II (30), growth factor (9), or mechanical stress (34). The signal transduction of cardiac growth including MAP kinase is initiated by the activation of the GTP-binding protein Ras. Activated Ras then phosphorylates Raf-1 and finally activates MAP kinases (30). Because simvastatin and atorvastatin significantly inhibited the MAP kinase activity in the LVFW, these drugs may prevent downstream reactions from this enzyme, resulting in reduction of transcription from DNA to RNA. This may be responsible for the decreases in RNA contents in the LVFW observed in the simvastatin- and atorvastatin-treated groups (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>SBP</th>
<th>DBP</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within 2 h after</td>
<td>6</td>
<td>57±2</td>
<td>38±2</td>
<td>175±9</td>
</tr>
<tr>
<td>6 Days after birth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle treated</td>
<td>7</td>
<td>94±6</td>
<td>55±3</td>
<td>202±18</td>
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<tr>
<td>Simvastatin treated</td>
<td>7</td>
<td>82±11</td>
<td>52±6</td>
<td>214±9</td>
</tr>
<tr>
<td>Atorvastatin treated</td>
<td>7</td>
<td>82±5</td>
<td>52±2</td>
<td>205±17</td>
</tr>
<tr>
<td>Pravastatin treated</td>
<td>9</td>
<td>84±4</td>
<td>52±3</td>
<td>180±7</td>
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</table>

Values are means ± SE; n = no. of observations. The piglets were used within 2 h after birth or 6 days after oral administration of either vehicle, simvastatin (2 mg·kg⁻¹·day⁻¹), atorvastatin (2 mg·kg⁻¹·day⁻¹), or pravastatin (4 mg·kg⁻¹·day⁻¹). The arterial systolic (SBP) and diastolic blood pressures (DBP) were measured via a cannula inserted into the left carotid artery. Heart rate (HR) was counted from electrocardiogram limb lead II. *P < 0.05 compared to piglets within 2 h after birth.
Farnesyl or geranylgeranyl groups should be covalently linked to Ras and the Ras-related family to facilitate heart growth (29). Prevention of isoprenoid formation by a lipophilic HMG-CoA reductase inhibitor may lead to decreased MAP kinase activity and limit heart growth just after birth. Li et al. (15) reported that lovastatin decreases protein isoprenylation and shifts the distribution of several small GTP-binding proteins from the membrane to the cytosolic fraction in HIT-T15 cells. As a result, the drug attenuates potentiation of nutrient-induced insulin secretion from the cell induced by bombesin and vasopressin (16). We tried but unfortunately failed to confirm the localization of Ras proteins between the membrane and cytosol in the piglet myocardium because the amount of the protein was negligible in nature. Further studies are needed to elucidate whether a lipophilic HMG-CoA reductase inhibitor affects the translocation of isoprenylated proteins including Ras.

Although simvastatin and atorvastatin interfered with the cardiac myocyte growth in neonatal piglets after birth, we did not find any significant changes in blood pressure and heart rate compared with those in the vehicle-treated group. The blood pressure could be maintained by some compensatory mechanisms, such as an increase in the total peripheral resistance, even when the left ventricle growth was limited.

Although the molecular mechanisms remain to be determined, the present study suggests that simvastatin and atorvastatin, lipophilic HMG-CoA reductase inhibitors, interfere with heart growth, which accounts for the myocyte cellular hypertrophy in the LVFW of neonatal piglets after birth. Pravastatin, a hydrophilic inhibitor, does not show any effect on heart growth in neonatal piglets.

Simvastatin, atorvastatin, and sodium pravastatin were kindly supplied by Sankyo Company Limited, Tokyo, Japan.

REFERENCES


