Simvastatin Inhibits Catecholamine Secretion and Synthesis Induced by Acetylcholine via Blocking Na\(^+\) and Ca\(^{2+}\) Influx in Bovine Adrenal Medullary Cells

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Received April 2, 2008; accepted June 30, 2008

**ABSTRACT**

Simvastatin, an inhibitor of HMG-CoA reductase, is a potent inhibitor of cholesterol biosynthesis and has beneficial effects in the primary and secondary prevention of cardiovascular diseases. In this study, we report the effects of simvastatin on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells used as a model of sympathetic neurons. Simvastatin inhibited catecholamine secretion induced by acetylcholine, an agonist of the nicotinic acetylcholine receptor; by veratridine, an activator of voltage-dependent Na\(^+\) channels; and by high K\(^+\), an activator of voltage-dependent Ca\(^{2+}\) channels (IC\(_{50}\) = 3.8, 7.8, and 6.1 \(\mu M\), respectively). Simvastatin also suppressed acetylcholine-induced \(^{22}\)Na\(^+\) influx (IC\(_{50}\) = 4.3 \(\mu M\)) and \(^{45}\)Ca\(^{2+}\) influx (IC\(_{50}\) = 6.1 \(\mu M\)), veratridine-induced \(^{22}\)Na\(^+\) influx (IC\(_{50}\) = 6.6 \(\mu M\)) and \(^{45}\)Ca\(^{2+}\) influx (IC\(_{50}\) = 12 \(\mu M\)), and high K\(^-\) -induced \(^{45}\)Ca\(^{2+}\) influx (IC\(_{50}\) = 11 \(\mu M\)). The reduction of catecholamine secretion caused by simvastatin was not overcome by increasing the concentration of acetylcholine or by treatment with mevalonate, the first metabolite of HMG-CoA. The inhibitory effect of simvastatin on histamine-induced secretion of catecholamines was observed in the presence of extracellular Ca\(^{2+}\), but not in a Ca\(^{2+}\)-free medium, suggesting that simvastatin does not interfere with histamine receptors nonselectively. Simvastatin also suppressed acetylcholine-induced \(^{[14]}\)Ccatecholamine synthesis from \(^{[14]}\)Ctyrosine as well as tyrosine hydroxylase activity. These findings suggest that simvastatin inhibits catecholamine secretion and synthesis induced by acetylcholine through suppression of Na\(^+\) and Ca\(^{2+}\) influx in the adrenal medulla and probably in the sympathetic neurons.

Simvastatin has been reported to have preventive effects on the process of atherosclerosis, improving the outcome among patients with chronic heart failure (Kjekshus et al., 1997) and reducing deaths caused by myocardial infarction and stroke (Pedersen et al., 2000). The molecular mechanism of the action of statins is the inhibition of enzyme HMG-CoA reductase, which catalyzes the formation of mevalonate, a precursor of isoprenoids, and thereby of cholesterol (Goldstein and Brown, 1984). The positive effects of statin therapy were initially attributed to a reduction in serum cholesterol; however, in recent years it has become increasingly obvious that statins have several other effects not directly related to their cholesterol-reducing action, namely, their so-called pleiotropic effects. These effects include improving endothelial function (Laufs et al., 1998), enhancing stability of atherosclerotic plaques (Fukumoto et al., 2001), decreasing oxidative stress (Stoll et al., 2005) and inflammation (Ridker et al., 1998, 2001), and inhibiting the thrombogenic response (Notarbartolo et al., 1995). In addition to these pleiotropic effects, Pliquett et al. (2003) reported that simvastatin lowered the plasma concentration of norepinephrine in rabbits associated with congestive heart failure. Plasma epinephrine concentration was slightly lower in patients treated with simvastatin therapy (Nette et al., 2005). Although an endogenous excess of catecholamines may be a risk factor for the development of atherosclerosis (Westfall and Westfall, 2005), it is not clear whether simvastatin affects the function of sympathetic neurons and the adrenal medulla.

Adrenal medullary cells (derived from the embryonic neural crest) are functionally homologous to the sympathetic postganglionic neuronal cells; both cells increase the secretion and synthesis of catecholamines in response to stimula-
tion of acetylcholine receptors (AChRs). In cultured bovine adrenal medullary cells, our previous study (Wada et al., 1985) showed that either carbachol-induced $^{22}\text{Na}^{+}$ influx via nicotinic AChRs (nAChRs)-ion channels, or veratridine-induced $^{22}\text{Na}^{+}$ influx via voltage-dependent Na$^{+}$ channels increases $^{45}\text{Ca}^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels, a prerequisite for the secretion (Wada et al., 1985) and synthesis (Yanagihara et al., 1987) of catecholamines; in contrast, high K$^{+}$ directly gates voltage-dependent Ca$^{2+}$ channels to increase $^{45}\text{Ca}^{2+}$ influx without increasing $^{22}\text{Na}^{+}$ influx. Because catecholamine secretion mediated by activation of these ion channels and the mechanism of stimulation of catecholamine synthesis in adrenal medullary cells are thought to be similar to those of norepinephrine in the sympathetic neurons, adrenal medullary cells have provided a good model for the detailed analysis of the action of cardiovascular drugs such as natriuretic peptides (Yanagihara et al., 1991), carvedilol (Kajiwara et al., 2002), and pimobendan (Toyohira et al., 2005).

In the present study, to examine the effects of simvastatin on nAChR-ion channels, voltage-dependent Na$^{+}$ channels, and voltage-dependent Ca$^{2+}$ channels, we investigated the direct effects of simvastatin on $^{22}\text{Na}^{+}$ influx, $^{45}\text{Ca}^{2+}$ influx, and catecholamine secretion induced by various secretagogues, such as acetylcholine, veratridine, and high K$^{+}$, using cultured bovine adrenal medullary cells. We have also examined the effect of simvastatin on catecholamine synthesis and the activity of tyrosine hydroxylase, the rate-limiting step of catecholamine biosynthesis.

Materials and Methods

Materials. Simvastatin, (+)-1S, 3R, 7S, 8S, 8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl 8-[2-[(2R, 4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl][ethyl]-1-naphthyl-2,2-dimethylbutanoyl, was kindly donated from Merck (Whitehouse Station, NJ). Other reagents were obtained from the following sources: Eagle's minimal essential medium, Nissui Pharmaceutical (Tokyo, Japan); calf serum and histamine, Nacalai Tesque (Kyoto, Japan); collagenase, Nitta Zerachin (Osaka, Japan); acetylcholine, veratridine, and mevalonolactone, Sigma-Aldrich (St. Louis, MO); [45]Cl and L-[U-14C]tyrosine, GE Healthcare (Little Chalfont, Buckinghamshire, UK); and [22]NaCl and [24]wells in a 5% CO$_2$, 95% air. The cells were used for experiments between 2 and 5 days of culture. Catecholamine Secretion. Oxygenated Krebs-Ringer phosphate (KRP) buffer (153 mM NaCl, 5.6 mM KCl, 1.1 mM MgSO$_4$, 2.2 mM CaCl$_2$, 0.85 mM Na$_2$HPO$_4$, 2.15 mM Na$_2$PO$_4$, and 10 mM glucose, adjusted to pH 7.4) was used throughout, except if otherwise specified. Cells were incubated with or without simvastatin in the presence or absence of various secretagogues at 37°C for 10 min. After the reaction, the incubation medium was transferred immediately to a test tube containing perchloric acid (final concentration, 0.4 M). Catecholamines (norepinephrine and epinephrine) secreted into the medium were adsorbed onto aluminum hydroxide and estimated by the ethylenediamine condensation method using a fluorescence spectrophotometer (F-4010; Hitachi, Tokyo, Japan) with excitation and emission wavelengths of 420 and 540 nm, respectively. $^{22}\text{Na}^{+}$ Influx and $^{45}\text{Ca}^{2+}$ Influx. The influx of $^{22}\text{Na}^{+}$ and $^{45}\text{Ca}^{2+}$ was measured as reported previously (Wada et al., 1985). Cells were incubated with 1.5 μCi of $^{22}\text{NaCl}$ or 1.5 μCi of $^{45}\text{CaCl}_2$ at 37°C for 5 min with or without various secretagogues and simvastatin in KRP buffer. After incubation, the cells were washed with ice-cold KRP buffer, solubilized in 10% Triton X-100, and counted for radioactivity of $^{22}\text{Na}^{+}$ and $^{45}\text{Ca}^{2+}$ by an Aloka ARC-2005 gamma counter and a Beckman LS-7000 liquid scintillation counter, respectively. 14C-Catecholamine Synthesis from [14C]Tyrosine. Cells were incubated with 20 μM L-[U-14C]tyrosine (1.0 μCi) in KRP buffer in the presence or absence of various concentrations of simvastatin and/or 0.3 mM acetylcholine at 37°C for 20 min. After the incubation medium by aspiration, cells were harvested in 0.4 M perchloric acid and centrifuged at 1600g for 10 min. 14C-Labeled catechol compounds were separated further by ion exchange chromatography on Duolite C-25 columns (H⁺-type, 0.4 × 7.0 cm) and counted for radioactivity (Yanagihara et al., 1987). [14C]Catecholamine synthesis was expressed as the sum of the [14C]catecholamines (epinephrine, norepinephrine, and dopamine).

Tyrosine Hydroxylase Activity in Situ. The cells (10⁶ cells/well; 24-well plates; Corning Life Sciences, Lowell, MA) were incubated with or without simvastatin and/or acetylcholine, supplemented with 18 μM L-[1-14C]tyrosine (0.2 μCi) for 10 min at 37°C in KRP buffer. After addition of L-[1-14C]tyrosine, each well was sealed immediately with an acrylic tube capped with a rubber stopper to absorb 14CO2 released by the cells, and cells were counted for radioactivity (Yanagihara et al., 2005).

Expression of Acetylcholine Receptors in Xenopus laevis Oocytes and Electrophysiological Recordings. Isolation and microinjection of X. laevis oocytes were performed as described previously (Ueno et al., 2004). The cDNAs encoding the α3 and β4 subunits of rat neuronal nAChR, subcloned into pcDNA1/Neo (Invitrogen, Carlsbad, CA) vector, were kindly provided from Dr. James W. Patrick (Division of Neuroscience, Baylor College of Medicine, Houston, TX). Oocytes were injected with cDNAs (1.5 ng/30 nl), and electrophysiological recordings were performed 2 to 3 days after injection. Each oocyte was perfused (2 ml·min⁻¹) with Ba²⁺-Ringer’s solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl₂, and 10 mM HEPES, pH 7.4) containing 1 μM atropine sulfate, to minimize the effects of secondarily activated Ca²⁺-dependent Cl⁻ currents, and then it was impaled with two glass electrodes (1–5 MΩ) filled with 3 M KCl and clamped at −70 mV using the OC-725C Oocyte Clamp Amplifier (Harvard Apparatus Inc., Holliston, MA). Acetylcholine was applied for 30 s to obtain the maximal (peak) current used as a measure of drug response. We tested the capacity of simvastatin to modify the effect of a concentration of acetylcholine that produced 50% of the maximal effect (EC50) of acetylcholine. This EC50 (70–80 μM) was determined individually for each oocyte, using 1 mM acetylcholine to produce a maximal current. Simvastatin was first dissolved in dimethyl sulfoxide and then diluted in Ba²⁺-Ringer’s solution before use at a final dimethyl sulfoxide concentration not exceeding 0.05%, which had no effect on acetylcholine-evoked responses (data not shown). Simvastatin was preapplied for 2 min before application of acetylcholine to allow for complete equilibration of the oocytes with simvastatin. In all cases, a 10- to 15-min washout period was allowed after application of the simvastatin and acetylcholine solutions.

Statistical Analysis. All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as means ± S.E.M. The significance of differences between means was evaluated using Student’s t test or one-way analytical of variance. Values were considered statistically different when P < 0.05. Statistical analyses were performed using StatView for Macintosh version 5.0J software (Abacus Concepts, Berkeley, CA).
Results

Concentration-Inhibition Curves for the Effect of Simvastatin on Secretagogue-Induced Catecholamine Secretion and $^{22}\text{Na}^+$ and $^{45}\text{Ca}^{2+}$ Influx in Cultured Bovine Adrenal Medullary Cells. We examined the effect of simvastatin on catecholamine secretion and $^{22}\text{Na}^+$ and $^{45}\text{Ca}^{2+}$ influx by various secretagogues. Acetylcholine (300 nM), an agonist of nAChR-ion channels, caused the secretion of catecholamines (15.3 ± 0.3% of the total catecholamines). Simvastatin reduced acetylcholine-induced catecholamine secretion in a concentration-dependent manner (IC$_{50}$ = 3.8 μM; Fig. 1A). Significant inhibition induced by simvastatin was observed at 1.0 μM, and maximal inhibition at 100 μM. Simvastatin (10–100 μM) also reduced acetylcholine-induced $^{22}\text{Na}^+$ influx (IC$_{50}$ = 4.3 μM) and $^{45}\text{Ca}^{2+}$ influx (IC$_{50}$ = 6.1 μM) in a concentration-dependent manner similar to its effect on acetylcholine-induced catecholamine secretion (Fig. 1B and C). Simvastatin (100 μM) did not affect any basal responses of catecholamine secretion (control, 0.41 ± 0.08%; simvastatin, 0.53 ± 0.07%). Veratridine (0.1 mM), an activator of voltage-dependent Na$^+$ channels, increased the secretion of catecholamines (22.6 ± 0.4% of the total catecholamines). Simvastatin (1.0 or 10–100 μM) significantly inhibited veratridine-induced catecholamine secretion (IC$_{50}$ = 7.8 μM), $^{22}\text{Na}^+$ influx (IC$_{50}$ = 6.6 μM) and $^{45}\text{Ca}^{2+}$ influx (IC$_{50}$ = 12 μM) (Fig. 2, A–C). Simvastatin (10–100 μM) also significantly suppressed catecholamine secretion (IC$_{50}$ = 6.1 μM) and $^{45}\text{Ca}^{2+}$ influx (IC$_{50}$ = 11 μM) induced by 56 mM K$^+$, an activator of voltage-dependent Ca$^{2+}$ channels (Fig. 3, A and B).

Characterization of the Inhibitory Effect of Simvastatin on Acetylcholine-Induced Catecholamine Secretion. We determined whether simvastatin was competing with acetylcholine for binding sites on the nAChR. When the concentration of acetylcholine in the incubation medium increased, the inhibition of catecholamine secretion induced by simvastatin was not overcome by acetylcholine at concentrations of 3 to 300 μM (Fig. 4).

Effects of Simvastatin on Acetylcholine Responses in X. laevis Oocytes Expressing nAChRs. The direct effects of simvastatin on acetylcholine responses in X. laevis oocytes expressing rat α3β4 nAChRs were examined. As shown in Fig. 5, simvastatin inhibited acetylcholine-induced currents in a concentration-dependent manner, and a significant inhibition was observed at 1.0 to 30 μM of simvastatin (IC$_{50}$ = 5.3 μM).

Effects of Mevalonate on Simvastatin-Induced Inhibition of Catecholamine Secretion. We examined the effect of simvastatin on acetylcholine-evoked catecholamine secretion in the cell in the presence of mevalonolactone, which is nonenzymatically converted to mevalonate, the first metabolite of HMG-CoA, in the medium. Simvastatin reduced acetylcholine-induced catecholamine secretion in the presence as well as the absence of mevalonolactone (200 μM) (Fig. 6A).

Effects of Simvastatin on Histamine-Stimulated Catecholamine Secretion. To investigate the effect of simvastatin on the function of another type of receptor involved in the mobilization of intracellular Ca$^{2+}$, we examined the effect of simvastatin on catecholamine secretion induced by histamine (Stauder and Pruss, 1990). Simvastatin (100 μM) significantly inhibited histamine (100 μM)-stimulated catecholamine secretion in the presence of extracellular Ca$^{2+}$ (Fig. 6B). It also inhibited $^{45}\text{Ca}^{2+}$ influx induced by histamine (100 μM) (data not shown). In the absence of extracellular Ca$^{2+}$, histamine caused less catecholamine secretion than that in the presence of extracellular Ca$^{2+}$. However, simvastatin failed to inhibit histamine-induced catecholamine secretion in Ca$^{2+}$-free medium (Fig. 6B).

Effects of Simvastatin on Catecholamine Synthesis and Tyrosine Hydroxylase Activity in Cultured Bovine Adrenal Medullary Cells. We examined the effect of simvastatin on $^{[14]}\text{C}$catecholamine synthesis from $^{[14]}\text{C}$tyrosine in the cells. Acetylcholine (300 μM) increased the synthesis of $^{[14]}\text{C}$catecholamine synthesis by 197% over the control (Fig. 7A). Simvastatin (1.0–100 μM) significantly inhibited acetylcholine-induced $^{[14]}\text{C}$catecholamine synthesis concentration-dependently, whereas it reduced basal $^{[14]}\text{C}$catecholamine synthesis at only 100 μM.

We next examined the effect of simvastatin on tyrosine hydroxylase activity in the cells. Treatment of cells with simvastatin suppressed both basal and acetylcholine-induced tyrosine hydroxylase activity at concentrations similar to those for $^{[14]}\text{C}$catecholamine synthesis (Fig. 7B).
Discussion

Inhibitory Effect of Simvastatin on Catecholamine Secretion Mediated by Various Ion Channels. In the present study, we demonstrated that simvastatin at concentrations of 1.0 to 100 μM inhibits catecholamine secretion induced by acetylcholine, a physiological secretagogue, in adrenal medullary cells (Fig. 1A). Simvastatin also inhibited 22Na+ influx and 45Ca2+ influx induced by acetylcholone at concentrations similar to those inhibiting catecholamine secretion (Fig. 1B and C). Furthermore, the present study showed that simvastatin attenuates 22Na+ and/or 45Ca2+ influx and catecholamine secretion induced by veratridine (Fig. 2) and 56 mM K+ (Fig. 3), which activate voltage-dependent Na+ channels and Ca2+ channels, respectively. This inhibition by simvastatin of voltage-dependent Ca2+ channels is compatible with previous reports, showing that simvastatin reduced the L-type Ca2+ current in rat pancreatic β-cells (Yada et al., 1999) and in brain cerebral cortex of rats (Bergdahl et al., 2003). Our previous report showed that Na+ influx occurs via nAChR-ion channels or voltage-dependent Na+ channels; this stimulates the Ca2+ influx via voltage-dependent Ca2+ channels and evokes the secretion of catecholamines in adrenal medullary cells. In contrast, 56 mM K+ stimulates catecholamine secretion and Ca2+ influx via voltage-dependent Ca2+ channels without Na+ influx (Wada et al., 1985). Therefore, it is likely that simvastatin inhibits catecholamine secretion induced by acetylcholone, veratridine, and 56 mM K+ through nAChR-ion channels, voltage-dependent Na+ channels, and Ca2+ channels, respectively. To the best of our knowledge, this is the first evidence showing the inhibitory effects of simvastatin on catecholamine secretion, 22Na+ influx, and 45Ca2+ influx mediated through these three ion channels in adrenal medullary cells.

Inhibitory Mechanism by Simvastatin of Catecholamine Secretion. It is generally accepted that statins prevent the conversion of HMG-CoA to mevalonate and subsequently reduce the synthesis of cholesterol, which induces the main effect of statins. A recent study reported that in neonatal rat
cardiac myocytes, atorvastatin suppressed the stimulatory effect of isoprenaline on cyclic ACC accumulation, which was abolished by mevalonate, suggesting an HMG-CoA reductase-dependent pathway (Mühlhäuser et al., 2006). However, the present study demonstrated that mevalonolactone, which is nonenzymatically converted to mevalonate, the first metabolite of HMG-CoA, did not abolish the inhibitory effect of simvastatin on acetylcholine-induced catecholamine secretion (Fig. 6A). It is likely that the inhibitory effect of simvastatin on catecholamine secretion induced by acetylcholine is not due to its inhibition of mevalonate-derived isoprenoid synthesis.

Although pravastatin, a hydrophilic statin, as well as simvastatin and atorvastatin, lipophilic statins, are reported to inhibit mevalonate synthesis (Endo, 1992), in the present study pravastatin did not inhibit acetylcholine-induced catecholamine secretion in cultured adrenal medullary cells (T. Matsuda, Y. Toyohira, and N. Yanagihara, unpublished observations). Furthermore, several previous studies reported that lovastatin, a lipophilic statin, inhibited the L-type Ca²⁺ current and reduced the intracellular free Ca²⁺ concentration and contraction in the rat cerebral artery, but that pravastatin did not (Bergdahl et al., 2003), and that simvastatin, but not paravastatin, inhibited glucose-induced cytosolic Ca²⁺ signaling and insulin secretion due to blockade of L-type Ca²⁺ channels in rat islet β-cells (Yada et al., 1999). Based on these data, we reached the conclusion that the inhibitory potency of statins on ion channel activity may correlate with their hydrophobicity.

In our previous report (Yoshimura et al., 1995), we demonstrated that carbamazepine, a drug for the treatment of seizure disorder, preferentially inhibits N-type Ca²⁺ channels, using three inhibitors of voltage-dependent Ca²⁺ channels such as nitrendipine (for L-type), ω-agatoxin-IVA (for P-type), and ω-conotoxin-GVIA (for N-type) at the maximal concentration. In our preliminary experiment, we examined the effect of combination of simvastatin with various inhibitors of voltage-dependent Ca²⁺ channels on catecholamine secretion induced by 56 mM K⁺. The degree of inhibition observed with combination of simvastatin (10 μM = IC₅₀) with two inhibitors of the three, such as nitrendipine (10 μM), ω-conotoxin-GVIA (1.0 μM), or ω-agatoxin-IVA (300 nM), on 56 mM K⁺-induced secretion of catecholamines was larger than that observed with the two Ca²⁺ channel inhibitors alone (T. Matsuda, Y. Toyohira, and N. Yanagihara, unpublished observations). We also observed that Cd²⁺, a nonselective inhibitor of voltage-dependent Ca²⁺ channels (500 μM), greatly inhibited 56 mM K⁺-induced secretion. The combination of simvastatin (10 μM = IC₅₀) with Cd²⁺ did not produce any further inhibition, compared with that caused by Cd²⁺. Furthermore, 56 mM K⁺-induced secretion was decreased to approximately 20% by simvastatin (100 μM) alone (Fig. 3A), suggesting that simvastatin reduces 56 mM K⁺-induced secretion of catecholamines probably by inhibiting all types (L-, N-, and P-types) of Ca²⁺ channels in the cells.

The precise mechanism or site of action of simvastatin on ion channels remains to be determined. The inhibitory effect of simvastatin was not overcome when the concentration of acetylcholine was increased, suggesting that simvastatin does not compete with acetylcholine at nAChRs. In X. laevis oocytes expressing α3β4 nAChRs, simvastatin inhibited acetylcholine-induced Na⁺ currents in a concentration-dependent manner (IC₅₀ = 5.3 μM) (Fig. 5) similar to those of 22Na⁺ influx, 45Ca²⁺ influx, and catecholamine secretion (IC₅₀ = 4.3, 6.1, and 3.8 μM, respectively) induced by acetylcholine in adrenal medullary cells (Fig. 1). Among nAChRs, α3β4 and α3β4α5 are expressed in adrenal medulla and autonomic neurons, and they have been found in the brain (Picciotto et al., 2001; Di Angelantonio et al., 2003). The agonist-binding site was located at the interface between α3 and β4 subunits, and was mostly composed of aromatic residues (Costa et al., 2003). A previous review (Léna and Changeux, 1993) proposed that noncompetitive blockers such as phencyclidine inhibit nAChRs at the area of interface between the nAChR protein and the membrane phospholipids. These findings led us to hypothesize that simvastatin acts on nAChRs at a site different from acetylcholine-binding site, which is probably located in an area of hydrophobic regions or lipid-protein interface within the receptor-ion channel. Further study is required to examine this possibility.

To examine the effect of simvastatin on another receptor-mediated ion flux or mobilization of intracellular Ca²⁺, we used histamine, which induces Ca²⁺ influx from the medium and increases the formation of inositol triphosphate and subsequently Ca²⁺ mobilization from intracellular Ca²⁺ stores mediated through histamine H₁ receptors in bovine adrenal chromaffin cells (Stauderman and Pruss, 1990; Cheek et al., 1993). In the present study, simvastatin inhibited histamine-induced catecholamine secretion in the presence but not in the absence of extracellular Ca²⁺ (Fig. 6B). Using a Ca²⁺-imaging system with acetoxymethyl esters of fura-2, we also observed that histamine (10 μM) caused two phase increases in intracellular Ca²⁺ in the presence of extracellular Ca²⁺, i.e., the first rapid peak of increase in intracellular Ca²⁺ is mediated by Ca²⁺ mobilization from intracellular store sites and the late prolonged phase is mediated by Ca²⁺ influx from the incubation medium. Simvastatin (100 μM) suppressed histamine-induced increase in late prolonged phase of intracellular Ca²⁺ in the presence extracellular Ca²⁺, but not in the rapid peak of intracellular Ca²⁺ in the absence of extracellular Ca²⁺ (T. Matsuda, Y. Toyohira, and N. Yanagihara, unpublished observations), suggesting that simvastatin suppresses Ca²⁺ influx from the incubation medium, but not Ca²⁺ mobilization from intracellular store sites. These results are consistent with the data of catecholamine secretion induced by histamine. Thus, it is likely that simvastatin suppresses Ca²⁺ influx but not Ca²⁺ mobiliza-
tion from intracellular Ca\textsuperscript{2+} stores, suggesting that simvastatin does not interfere with histamine receptors nonselectively.

**Inhibition by Simvastatin of Catecholamine Synthesis.** In the present study, we showed that simvastatin inhibits not only catecholamine secretion but also catecholamine synthesis. Incubation of cells with simvastatin inhibited basal and acetylcholine-induced \textsuperscript{14}C|catecholamine synthesis from \textsuperscript{14}C|tyrosine in the cells (Fig. 7A). Furthermore, simvastatin suppressed basal and acetylcholine-induced tyrosine hydroxylase activity at concentrations similar to those at which \textsuperscript{14}C|catecholamine synthesis was inhibited (Fig. 7B). From these findings, it seems that simvastatin attenuates tyrosine hydroxylase activity, and subsequently suppresses catecholamine synthesis in the cells stimulated by acetylcholine, although it is not yet possible to know whether simvastatin preferentially reduces the synthesis or the secretion of catecholamines caused by acetylcholine.

**Clinical Implication of Simvastatin-Induced Inhibition of Catecholamine Synthesis and Secretion.** In the present study, we found that simvastatin at 1.0 µM significantly inhibited catecholamine synthesis and secretion induced by acetylcholine, a physiological secretagogue. However, the concentrations of simvastatin used in the present study may be higher than that in the plasma of individuals clinically treated with statins, which range between 0.01 and 0.1 (Laufs et al., 1998; Lilja et al., 1998) or 0.01 and 1.0 µM (Kantola et al., 1998). Simvastatin is lipophilic and administered daily for the treatment of hypercholesterolemia or atherosclerosis. Therefore, it is possible that chronic administration of simvastatin might accumulate in tissues, resulting in higher local concentrations than that reported in the plasma.

Activation of the sympathetic nervous system may participate in hypertension, left ventricular hypertrophy, and hyperlipemia. Evidence has accumulated that the aberrant chronic
activation of the sympathetic nervous system is involved causally in the development of chronic heart failure (Kaye et al., 1995; Freedman and Leffkowitz, 2004; Lympouropoulos et al., 2007). In the present study, we demonstrated the inhibitory effects of simvastatin on catecholamine synthesis and secretion, suggesting that the effect of simvastatin in clinical treatments is mediated in part by reduced plasma catecholamines through suppression of the sympathetic nervous activity and adrenal medullary functions. Indeed, statins have been demonstrated to have beneficial effects on cardiovascular diseases, lowering the incidence of new-onset heart failure, and improving outcomes among patients with chronic heart failure (Kjekshus et al., 1997). Furthermore, several lines of evidence have shown that statins reduce the level of plasma norepinephrine in chronic heart failure rabbits (Pliquett et al., 2003) and suppress 24-h urinary norepinephrine excretion in spontaneously hypertensive rats (Kishi et al., 2003). Taken together, simvastatin may improve the clinical symptoms of cardiovascular diseases through inhibition of catecholamine synthesis and release in the sympathetic neurons and adrenal medulla. To confirm this possibility, further in vivo administration of simvastatin to humans is required in future study.

In conclusion, simvastatin inhibits acetylcholine-induced Na+ influx and Ca2+ influx and subsequently reduces catecholamine secretion and catecholamine synthesis in cultured adrenal medullary cells in a manner independent of cholesteryl biosynthesis. This inhibitory effect of simvastatin on catecholamine signals may play a role in its pleiotropic effects on cardiovascular diseases.

Acknowledgments

We are grateful to Dr. James A. Patrick for providing rat acetylcholine receptor subunit cDNAs used in this study and Dr. Rintaro Nakamura for excellent technical assistance. We also thank Merck for the generous gift of simvastatin.

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sive rats (Kishi et al., 2003). Taken together, simvastatin may

by pimobendan, a drug for the treatment of heart failure: reduction of catechol-

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