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Lovastatin prevents angiotensin II-induced cardiac hypertrophy in cultured neonatal rat heart cells

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Abstract

Angiotensin II activates $p21^{ras}$, and mediates cardiac hypertrophic growth through the type 1 angiotensin II receptor in cardiac myocytes. An inhibitor of *3-hydroxy-3-methyglutaryl-coenzyme A* (HMG-CoA) reductase has been shown to block the post-translational farnesylation of $p21^{ras}$ and inhibit protein synthesis in several cell types. Primary cultures of neonatal cardiac myocytes were used to determine whether HMG-CoA reductase inhibitors, lovastatin, simvastatin and pravastatin inhibit the angiotensin II-induced hypertrophic growth. Angiotensin II (10^{-6} M) significantly increased protein–DNA ratio, RNA–DNA ratio, ratios of protein synthesis and mitogen-activated protein (MAP) kinase activity. Lipid-soluble HMG-CoA reductase inhibitors, lovastatin (10^{-6} M) and simvastatin (10^{-6} M) partially and significantly inhibited the angiotensin II-induced increases in these parameters, but a water-soluble HMG-CoA reductase inhibitor, pravastatin (10^{-6} M) did not. Mevalonate (10^{-4} M) overcame the inhibitory effects of lovastatin and simvastatin on angiotensin II-induced increases in these parameters. A selective protein kinase C inhibitor, calphostin C (10^{-6} M) partially and significantly prevented angiotensin II-induced increases in these parameters, and treatment with both lovastatin and calphostin C inhibited completely. Angiotensin II increased $p21^{ras}$ activity and membrane association, and lovastatin inhibited them. These studies demonstrate that a lipid-soluble HMG-CoA reductase inhibitor, lovastatin, may prevent angiotensin II-induced cardiac hypertrophy, at least in part, through $p21^{ras}$/MAP kinase pathway, which is linked to mevalonate metabolism.

Keywords: Cardiac myocyte; Hypertrophy; Angiotensin II; Cholesterol; Ras

1. Introduction

Mitogen-activated protein (MAP) kinases are a family of serine/threonine protein kinases activated as early responses to a variety of stimuli involved in cellular growth, transformation, and differentiation (Komuro et al., 1990; Morgan and Baker, 1991). Angiotensin II stimulates cellular hypertrophy through the type 1 angiotensin II receptor in cultured neonatal rat myocytes (Miyata and Haneda, 1994). It has been reported that MAP kinases are activated by angiotensin II or mechanical stress during cardiac myocyte hypertrophy through both protein kinase C-dependent and independent pathways (Yamazaki et al., 1995). On the other hand, Ras proteins constitutively activate the MAP kinase signaling pathway, thereby sending an uninterrupted growth signal (Gibbs, 1991). However, it is not well known how the activation of the Ras/Raf/MAP kinase pathway is involved in angiotensin II-induced cardiac hypertrophy in addition to activation of the protein kinase C/Raf/MAP kinase pathway.

The Ras proto-oncogenes encode closely relates proteins of relative molecular mass around 21,000 ($p21^{ras}$) (Gibbs, 1991). The activity of $p21^{ras}$ is regulated through binding of guanine nucleotides. Active $p21^{ras}$ is bound to GTP, whereas inactive $p21^{ras}$ is bound to GDP. $p21^{ras}$ must be localized in the plasma membrane after a series of post-translational modifications in order to function normally or lead to malignant transformation. The first and obligatory step in this series is farnesylation of the cysteine residue located at the $p21^{ras}$ COOH-terminal CAAX motif, where C is cysteine, A is any aliphatic amino acid, and X is methionine or serine (Willumsen et al., 1984). The addition of a farnesyl moiety to the $p21^{ras}$ CAAX peptide

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is catalyzed by farnesyl protein transferase. Therefore, farnesylation is critical for $p21^{ras}$'s membrane localization and function.

Thorburn et al. (1993) demonstrated the importance of the $p21^{ras}$/MAP kinase cascade in ventricular hypertrophy by direct needle microinjection of activated $p21^{ras}$ into primary neonatal rat ventricular cardiac myocytes. Recently, Sadoshima and Izumo (1993, 1996) have reported that mechanical loading or addition of angiotensin II rapidly activates $p21^{ras}$ in cultured neonatal rat cardiac myocytes. On the other hand, Hancock et al. (1989) have reported that inhibition of $p21^{ras}$ farnesylation interferes with its membrane localization and blocks $p21^{ras}$-mediated cellular transformation. It has been demonstrated that an inhibitor of 3-hydroxy-3-methyglutaryl-coenzyme A (HMG-CoA) reductase, which inhibits mevalonate synthesis, prevents $p21^{ras}$ farnesylation in *Xenopus* oocytes (Maltese et al., 1985) and suppresses the growth of murine tumor in vivo (Schafer et al., 1989). O'Donnell et al. (1993) have reported that lovastatin inhibits serum-induced proliferation of rat mesangial cells and that its inhibitory effect is completely prevented in the presence of exogenous mevalonate. There is a difference in the potency to inhibit mevalonate synthesis in extrahepatic cells between lipid-soluble HMG-CoA reductase inhibitors, such as lovastatin and simvastatin, and water-soluble HMG-CoA reductase inhibitors, such as pravastatin (Koga et al., 1992; Vliet et al., 1995).

However, it is not known whether HMG-CoA reductase inhibitors, which inhibit farnesylation of $p21^{ras}$, prevent cardiac hypertrophy. Therefore, to investigate whether HMG-CoA reductase inhibitors prevent cardiac hypertrophy through the $p21^{ras}$/MAP kinase pathway, we studied the effect of lovastatin on angiotensin II-induced hypertrophy of cultured neonatal rat heart myocytes and compared its effect with those of simvastatin and pravastatin.

2. Materials and methods

2.1. Myocyte culture

Monolayer cultures of 1- to 2-day old neonatal Sprague–Dawley rat (Japan SLC) myocytes were prepared with modifications as described previously (Haneda and McDermott, 1991). Minced ventricular myocardium was placed into Ca^{2+}- and Mg^{+2}-free Hanks' salt solution buffered with 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4. The cells were dissociated in a water-jacketed Celstir apparatus (Wheaton Scientific) at 37°C with a mixture of partially purified 2.4 IU/ml trypsin (Worthington Biochemical), 2.7 IU/ml α-chymotrypsin, and 0.94 IU/ml elastase (Sigma). After each of five successive 20-min incubations, the dissociated cells were mixed with Eagle's minimal essential medium (MEM) (Gibco) containing 10% newborn calf serum and were centrifuged and pooled. The dissociated cells were enriched for cardiomyocytes by the technique of differential adhesion for 90 min and plated at a concentration of 10^6 cells/60 mm dish. Cultures were incubated in a humidified 5% CO_2–95% air atmosphere at 37°C. After an overnight incubation in MEM containing 10% newborn calf serum and 0.1 mM 5-bromo-2′-deoxyuridine (Sigma), the attached cells were rinsed and maintained in serum-free medium. Briefly, standard MEM was supplemented with MEM amino acids, vitamins, penicillin–streptomycin (Gibco), and 2 mM glutamine. In addition, the medium contained 30 nM $NaSeO_4$, 2.5 μg/ml human insulin, 10 μg/ml human transferrin (Sigma), 0.25 mM ascorbic acid (Sigma), 10 μg/ml cholesterol (Sigma) and 0.1 mM 5-bromo-2′-deoxyuridine to minimize the proliferation of non-myogenic cells. The medium was replaced every 2 days with fresh medium over the time course of the experiments. The number of myocytes per culture dish did not change during angiotensin II treatment.

Stock solutions of angiotensin II (Sigma) was prepared in MEM at a concentration of 10^{-3} M, and stored at −20°C. Lovastatin (a gift of Merck) or simvastatin (a gift of Banyu Pharma) was dissolved in ethanol at a concentration of 7.5×10^{-3} or 10^{-3} M, respectively, and stored at −80 or −20°C, respectively. Pravastatin (a gift of Sankyo Pharma) was prepared in distilled water at a concentration of 10^{-3} M and stored at −80°C. A product of the HMG-CoA reductase reaction, mevalonate (Sigma) was prepared in distilled water at a concentration of 10^{-2} M, and stored at 4°C. A selective protein kinase inhibitor, calphostin C (Kobayashi et al., 1989) (Kyowa Hakko), was prepared in MEM at a concentration of 10^{-3} M, and stored at 4°C. The inhibitors were diluted in culture medium at the time of use.

2.2. Myocyte protein, DNA, and RNA content

Each individual culture dish was rinsed three times with ice-cold phosphate-buffered saline (PBS). The cell layer was scraped from each dish with two 0.5-ml volumes of 1× standard sodium citrate containing 0.25% (wt./vol.) sodium dodecyl sulfate (SDS) and frozen at −80°C. Before assay, the extracts were thawed and vortexed extensively. Protein content was assayed directly in aliquots of each extract by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. DNA content was measured fluorometrically in aliquots of each extract using calf thymus DNA as a standard (Cesarone et al., 1979). For RNA determination, a 75-μl aliquot from each extract was precipitated with an equal volume of ice-cold 1 N $HClO_4$. The material was centrifuged, and the pellet was washed three times with 0.5 N $HClO_4$. The pellet was hydrolyzed in 0.3 N NaOH for 24 h at 37°C, and the protein was reprecipitated by adding 4 N $HClO_4$ to a final concentration of 1 N. The supernatant was used to measure the absorbance at 260 and 232 nm following

centrifugation. The RNA concentration (μg/ml) was calculated according to the following equation (Munro and Fleck, 1966)

$$\text{RNA} = (A_{260} \times 32.9) - (A_{232} \times 6.11)$$

where A_{260} and A_{232} are the absorbances at 260 and 232 nm, respectively.

2.3. Protein synthesis

The rates of protein synthesis were measured at 2 days after the addition of angiotensin II. The amino acid incorporation was used as an index of the rates of protein synthesis with the assumption that the relationship between the specific radioactivity of phenylalanine tRNA and the specific radioactivity of extracellular phenylalanine was not modified by any drug. The relative amount of protein synthesis was determined by assessing the incorporation of radioactivity into a 10% trichloroacetic acid insoluble fraction after L-[^{14}C]phenylalanine (1 μCi/ml, Amersham) exposure. In the determination of the specific activity of the labeled protein, cells were rapidly rinsed three times with ice-cold PBS, and the protein was precipitated with 1 ml of 10% trichloroacetic acid for 1 h on ice and extracted overnight with 1 N NaOH (Morgan et al., 1971; McDermott and Morgan, 1989). After reprecipitation, the protein was used for determinations of radioactivity and protein concentration. Radioactivity was assessed using a liquid scintillation counter (Beckman). Cell protein was determined by the method of Lowry et al. (1951).

2.4. MAP kinase activity

The MAP kinase activities were measured by phosphocellulose binding paper assay format (p42/p44 MAP kinase enzyme assay system, Amersham) (Shirakabe et al., 1992). Cultured cardiac myocytes were rinsed three times with ice-cold Hanks' balanced salt solution buffer and scraped into 0.5 ml of ice-cold lysis buffer (10 mM Tris, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EGTA), 2 mM DL-dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.4). The cells were centrifuged at $25{,}000 \times g$ for 20 min. The supernatant was used to measure MAP kinase activity. Aliquots from each extract were incubated with 1 μCi [γ-^{32}P]ATP and 10 μl of substrate buffer (75 mM HEPES, 0.3 mM sodium orthovanadate, 0.05% sodium azide, pH 7.4) at 30°C for 30 min. The mixtures were transferred onto filter paper squares. The squares were washed with two changes of 75 mM orthophosphoric acid and with two changes of distilled water, and then were put into counting vials containing 10 ml scintillation solution. The radioactivity was counted using a liquid scintillation counter (Beckman). Cell protein was determined by the method of Lowry et al. (1951).

2.5. $p21^{ras}$ activity

The analysis of p21^{ras}-associated guanine nucleotides was performed by an immunoprecipitation assay as described previously (Downward et al., 1990). Cultured cardiac myocytes were labeled with [^{32}P]orthophosphate (0.1 mCi/ml, Nen) in phosphate-free Dulbecco's modified eagle medium (Gibco) for 8 h. Myocytes were lysed with lysis buffer (20 mM Tris, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 0.5% Triton X-100, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin). The cells were centrifugated at $500 \times g$ for 5 min to remove nuclei. The supernatant was subjected to immunoprecipitation with the monoclonal antibody to v-H-ras (Calbiochem) precoupled to protein A–agarose (Y13–259) for 60 min. Nucleotides were eluted with 20 mM Tris, 20 mM EDTA, 2% SDS at 65°C for 5 min and separated in 1 N KH_2PO_4 by PEI-cellulose thin-layer chromatography. After autoradiography, the intensities of blots were quantitated as photo-stimulated luminescence values by Bio-Image Analyzer System (Fujix), and ratios of GTP-bound p21^{ras}/GDP-bound p21^{ras} + GTP-bound p21^{ras} were determined as p21^{ras} activities.

2.6. $p21^{ras}$ membrane association

The analysis of membrane p21^{ras} was performed by Western blot analysis as described previously (Ohmura et al., 1993). Myocytes were lysed with lysis buffer (20 mM Tris, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 0.5% Triton X-100, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin). Cells were centrifugated at $500 \times g$ for 10 min, and the supernatant was recentrifugated at $100{,}000 \times g$ for 60 min. The resupernatant was used to measure p21^{ras} content in membrane fraction. For Western blot analysis, 100 μg of protein was subjected to 15% SDS–polyacrylamide gel (SDS–PAGE), and were then transferred to nitrocellulose membrane. The blots were incubated with the monoclonal antibody to v-H-ras (Calbiochem). Positive antibody reactions were visualized using by the horseradish peroxidase conjugated anti-rat antibody and the enhanced chemiluminescence detection system (ECL; Amersham).

2.7. Statistical analysis

All values are expressed as mean ± S.E.M. Statistical significance was determined using unpaired Student's *t*-test or multiple comparison test (Bonferroni/Dunn). The results were considered to be significant when $P < 0.05$.

3. Results

3.1. Effects of angiotensin II and HMG-CoA reductase inhibitors on cellular hypertrophy

Subconfluent neonatal rat heart cells in culture were used to determine the effects of angiotensin II on RNA content and myocyte growth. Angiotensin II was added to a final concentration of 10^{-6} M on culture day 4, and protein-to-DNA and RNA-to-DNA ratios were measured at 24 h intervals over the next 3 days in culture. The protein-to-DNA ratio increased over the first 2 days after the addition of angiotensin II (Fig. 1a). The protein-to-DNA ratios in angiotensin II-treated myocytes were increased significantly by 14 and 22% compared with controls at days 6 and 7, respectively. The RNA-to-DNA ratios increased significantly by 24% compared with controls 1 day after addition of angiotensin II (Fig. 1b). However, a decline of the RNA-to-DNA ratios occurred after 2 days of angiotensin II treatment. These findings indicated that angiotensin II rapidly increased the capacity for protein synthesis, as measured by RNA content, and that greater capacity was followed by accelerated myocyte growth.

In the next experiments, the effects of HMG-CoA reductase inhibitors, lovastatin, simvastatin and pravastatin, on angiotensin II-stimulated increases in protein-to-DNA and RNA-to-DNA ratios were studied to determine whether $p21^{ras}$, which is linked to mevalonate metabolism, was involved in angiotensin II-mediated hypertrophic growth. A HMG-CoA reductase inhibitor, lovastatin, simvastatin or pravastatin, was added 24 h before exposure to angiotensin II. Lovastatin (10^{-7} and 10^{-6} M) significantly inhibited the angiotensin II-induced increases in protein-to-DNA ratios at culture day 6 by 27 and 43%, respectively, and in RNA-to-DNA ratios at culture day 4 by 28 and 38%, respectively (Table 1). Simvastatin (10^{-7} and 10^{-6} M) significantly inhibited the angiotensin II-induced increases in protein-to-DNA ratios at culture day 6 by 37 and 52%, respectively, and in RNA-to-DNA ratios at culture day 4 by 33 and 53%, respectively (Table 2). The inhibitory effect of lovastatin or simvastatin on the angiotensin II-induced increases in protein-to-DNA and RNA-to-DNA ratios was completely prevented in the presence of mevalonate (10^{-4} M) (Tables 1 and 2). However, pravastatin did not inhibit the angiotensin II-induced increases in protein-to-DNA ratios at culture day 7 and in RNA-to-DNA ratios at culture day 5 (Table 3). Lovastatin, simvastatin or pravastatin, alone did not change values of protein-to-DNA and RNA-to-DNA ratios (Tables 1–3).

3.2. Effects of angiotensin II and HMG-CoA reductase inhibitors on protein synthesis

To determine whether the increase in cellular protein was the result of an increase in the rates of protein synthesis, rates of amino acid incorporation were determined by [^{14}C]phenylalanine incorporation into total protein (dpm/μg protein). Cultures of neonatal rat myocytes were pulsed with [^{14}C]phenylalanine for 2 h to determine rates of protein synthesis. Rates of protein synthesis were measured at 2 days after the addition of angiotensin II, when rapid increases in protein-to-DNA ratios were observed in this study (Fig. 1a).

Angiotensin II significantly increased the rates of protein synthesis by 15–17% (Tables 1–3). In the next experiments, the effects of HMG-CoA reductase inhibitors to prevent the acceleration of protein synthesis rates by angiotensin II was studied. A HMG-CoA reductase inhibitor was added first, followed 24 h later by angiotensin II. Lovastatin (10^{-7} and 10^{-6} M) or simvastatin (10^{-7} and 10^{-6} M) significantly inhibited the angiotensin II-induced increases in rates of protein synthesis by 25 and 41%, or 39 and 57%, respectively (Tables 1 and 2). The inhibitory

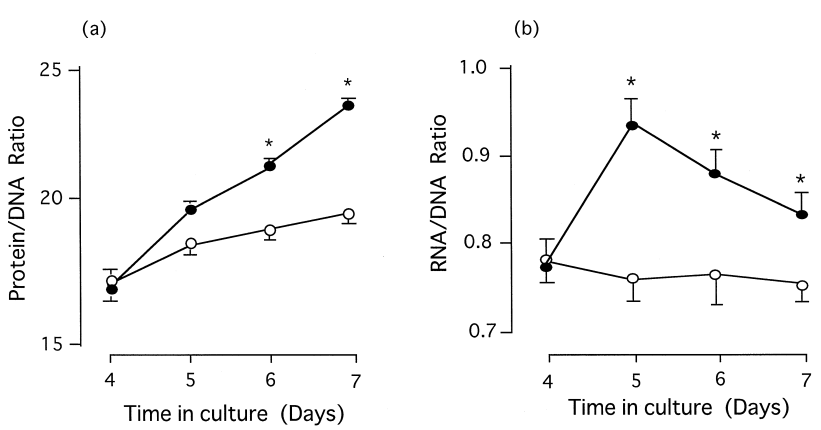

Fig. 1. Effects of angiotensin II on cell growth and RNA content. Values are means ± S.E.M. of eight dishes in four different preparations. Cardiomyocytes were incubated in serum-free medium and angiotensin II was added to a final concentration of 10^{-6} M on day 4. The ratios of total protein-to-DNA (a) and RNA-to-DNA (b) were measured at the indicated time points. ○ Control cells; ● angiotensin II-treated cells. Statistical comparison was made using unpaid Student's t-test. $P < 0.05$, compared with control cells at the same point.

Table 1
Effects of lovastatin and mevalonate on angiotensin II-induced hypertrophic growth
Values are means ± S.E.M. of 10 dishes in five different preparations. Cardiomyocytes were incubated in serum-free medium and angiotensin II was added to a final concentration of 10^{-6} M on day 4. Lovastatin or mevalonate was added 24 h before exposure to angiotensin II. The ratios of protein-to-DNA, the ratios of RNA-to-DNA and rates of protein synthesis were measured at day 7, 5 and 6, respectively. Statistical comparison was made using multiple comparison test.

Angiotensin II (M)	Lovastatin (M)	Mevalonate (M)	Protein-to-DNA	RNA-to-DNA	Rates of protein synthesis (dpm/μg protein)
			20.2 ± 0.4	0.75 ± 0.02	69.2 ± 0.7
10^{-6}			24.6 ± 0.4[a]	0.93 ± 0.01[a]	80.9 ± 1.0[a]
10^{-6}	10^{-7}		23.4 ± 0.3[a,b]	0.88 ± 0.01[a,b]	78.2 ± 0.6[a,b]
10^{-6}	10^{-6}		22.7 ± 0.3[a,b]	0.86 ± 0.01[a,b]	76.1 ± 0.8[a,b]
10^{-6}	10^{-6}	10^{-4}	24.5 ± 0.2[a,c]	0.93 ± 0.01[a,c]	81.0 ± 0.6[a,c]
	10^{-6}		20.2 ± 0.4	0.74 ± 0.01	69.8 ± 0.3
		10^{-4}	20.3 ± 0.4	0.76 ± 0.01	69.9 ± 0.8

[a] $P < 0.05$, compared with control cells at the same point.
[b] $P < 0.05$ compared with angiotensin II-treated cells at the same point.
[c] $P < 0.05$, compared with angiotensin II- and lovastatin (10^{-6} M)-treated cells at the same point.

Table 2
Effects of simvastatin and mevalonate on angiotensin II-induced hypertrophic growth
Values are means ± S.E.M. of 10 dishes in five different preparations. Cardiomyocytes were incubated in serum-free medium and angiotensin II was added to a final concentration of 10^{-6} M on day 4. Simvastatin or mevalonate was added 24 h before exposure to angiotensin II. The ratios of protein-to-DNA, the ratios of RNA-to-DNA and rates of protein synthesis were measured at day 7, 5 and 6, respectively. Statistical comparison was made using multiple comparison test.

Angiotensin II (M)	Simvastatin (M)	Mevalonate (M)	Protein-to-DNA	RNA-to-DNA	Rates of protein synthesis (dpm/μg protein)
			20.4 ± 0.4	0.73 ± 0.02	69.2 ± 0.8
10^{-6}			24.5 ± 0.4[a]	0.94 ± 0.01[a]	81.0 ± 1.0[a]
10^{-6}	10^{-7}		23.0 ± 0.2[a,b]	0.87 ± 0.01[a,b]	76.8 ± 0.7[a,b]
10^{-6}	10^{-6}		22.4 ± 0.5[a,b]	0.82 ± 0.01[a,b]	74.3 ± 0.8[a,b]
10^{-6}	10^{-6}	10^{-4}	24.4 ± 0.3[a,c]	0.94 ± 0.01[a,c]	81.1 ± 0.9[a,c]
	10^{-6}		20.3 ± 0.6	0.72 ± 0.02	69.2 ± 0.9
		10^{-4}	20.3 ± 0.3	0.74 ± 0.01	69.7 ± 0.9

[a] $P < 0.05$, compared with control cells at the same point.
[b] $P < 0.05$ compared with angiotensin II-treated cells at the same point.
[c] $P < 0.05$, compared with angiotensin II- and simvastatin (10^{-6} M)-treated cells at the same point.

Table 3
Effects of pravastatin on angiotensin II-induced hypertrophic growth
Values are means ± S.E.M. of 10 dishes in five different preparations. Cardiomyocytes were incubated in serum-free medium and angiotensin II was added to a final concentration of 10^{-6} M on day 4. Pravastatin was added 24 h before exposure to angiotensin II. The ratios of protein-to-DNA, the ratios of RNA-to-DNA and rates of protein synthesis were measured at day 7, 5 and 6, respectively. Statistical comparison was made using multiple comparison test.

Angiotensin II (M)	Pravastatin (M)	Protein-to-DNA	RNA-to-DNA	Rates of protein synthesis (dpm/μg protein)
		20.2 ± 0.3	0.76 ± 0.02	70.2 ± 0.8
10^{-6}		24.8 ± 0.4[a]	0.94 ± 0.04[a]	80.9 ± 0.9[a]
10^{-6}	10^{-7}	24.9 ± 0.4[a]	0.94 ± 0.03[a]	80.9 ± 0.8[a]
10^{-6}	10^{-6}	24.7 ± 0.5[a]	0.95 ± 0.01[a]	80.5 ± 0.9[a]
10^{-6}	10^{-5}	25.0 ± 0.5[a]	0.92 ± 0.01[a]	80.5 ± 0.6[a]
	10^{-5}	20.3 ± 0.3	0.75 ± 0.03	70.8 ± 0.9

[a] $P < 0.05$, compared with control cells at the same point.

Table 4
Effects of lovastatin and calphostin C on angiotensin II-induced hypertrophic growth
Values are means ± S.E.M. of eight dishes in four different preparations. Cardiomyocytes were incubated in serum-free medium and angiotensin II was added to a final concentration of 10^{-6} M on day 4. Lovastatin or calphostin C was added 24 h or 30 min before exposure to angiotensin II, respectively. The ratios of protein-to-DNA, the ratios of RNA-to-DNA and rates of protein synthesis were measured at day 7, 5 and 6, respectively. Statistical comparison was made using multiple comparison test.

Angiotensin II (M)	Lovastatin (M)	Calphostin C (M)	Protein-to-DNA	RNA-to-DNA	Rates of protein synthesis (dpm/μg protein)
			20.3 ± 0.3	0.74 ± 0.02	69.2 ± 0.6
10^{-6}			24.5 ± 0.4^{a}	0.94 ± 0.01^{a}	81.0 ± 0.8^{a}
10^{-6}	10^{-6}		$22.8 \pm 0.2^{a,b}$	$0.86 \pm 0.02^{a,b}$	$76.4 \pm 0.6^{a,b}$
10^{-6}		10^{-6}	$21.5 \pm 0.2^{a,b}$	$0.80 \pm 0.01^{a,b}$	$72.1 \pm 0.8^{a,b}$
10^{-6}	10^{-6}	10^{-6}	$20.1 \pm 0.4^{b,c,d}$	$0.75 \pm 0.02^{b,c,d}$	$70.1 \pm 0.5^{b,c,d}$
	10^{-6}		20.2 ± 0.3	0.73 ± 0.02	69.1 ± 0.5
		10^{-6}	20.0 ± 0.4	0.75 ± 0.01	68.9 ± 0.3

[a] $P < 0.05$, compared with control cells at the same point.
[b] $P < 0.05$ compared with angiotensin II-mediated cells at the same point.
[c] $P < 0.05$, compared with angiotensin II- and lovastatin-treated cells at the same point.
[d] $P < 0.05$, compared with angiotensin II- and calphostin C-treated cells at the same point.

effect of lovastatin or simvastatin on the angiotensin II-induced increases in rates of protein synthesis was completely prevented in the presence of mevalonate (Tables 1 and 2). However, pravastatin did not inhibit the angiotensin II-induced increases in rates of protein synthesis (Table 3). Lovastatin, simvastatin or pravastatin, alone did not change values of rates of protein synthesis (Tables 1–3).

3.3. Effects of angiotensin II, lovastatin and calphostin C on cellular hypertrophy

The effects of lovastatin and a selective protein kinase C inhibitor, calphostin C, on angiotensin II-induced increases in protein-to-DNA ratios, RNA-to-DNA ratios and *rates* of protein synthesis were studied to determine how the protein kinase C–MAP kinase pathway and $p21^{ras}$–MAP kinase pathway were involved in angiotensin II-induced hypertrophic growth. Calphostin C (10^{-6} M) was added 30 min before exposure to angiotensin II. Calphostin C significantly inhibited the angiotensin II-induced increases in protein-to-DNA ratios, RNA-to-DNA ratios and rates of protein synthesis by 71, 73 and 75%, respectively (Table 4). Furthermore, pretreatment with both lovastatin (10^{-6} M) and calphostin C completely inhibited the angiotensin II-induced increases in these parameters (Table 4). Calphostin C alone did not change values in these parameters (Table 4).

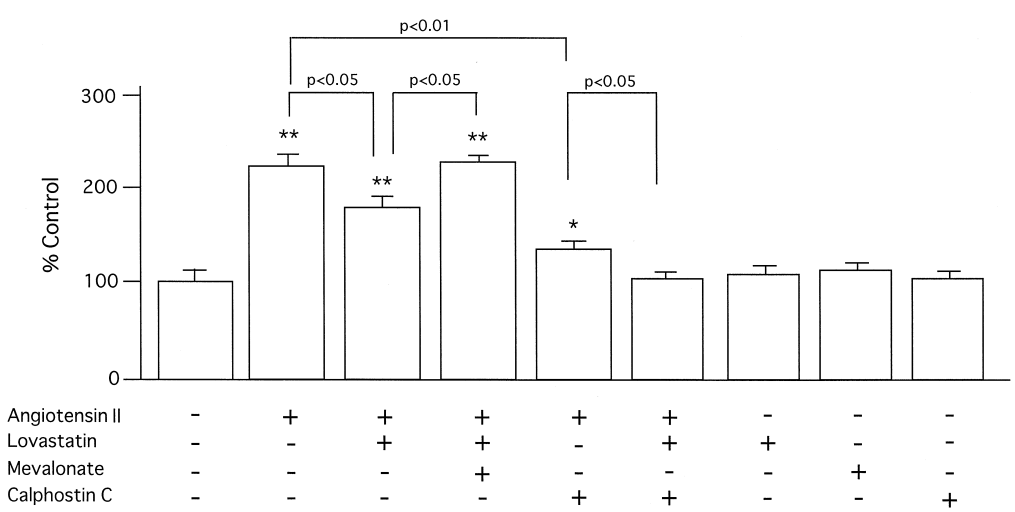

Fig. 2. Effects of angiotensin II, lovastatin and calphostin C on MAP kinase activity. MAP kinase activity was assayed 8 min after exposure of angiotensin II (10^{-6} M). Lovastatin (10^{-6} M), mevalonate (10^{-4} M) or calphostic C (10^{-6} M) was added 24 h, 24 h or 30 min before exposure of angiotensin II, respectively. Values at each time point are means ± S.E.M. of 10 dishes in five different preparations, and are percent changes compared with 0 min in each group. Statistical comparison within each treatment group or between treatment groups was made using unpaired Student's t-test or multiple comparison test, respectively. $*P < 0.05$, $**P < 0.01$, compared with control cells at the same point.

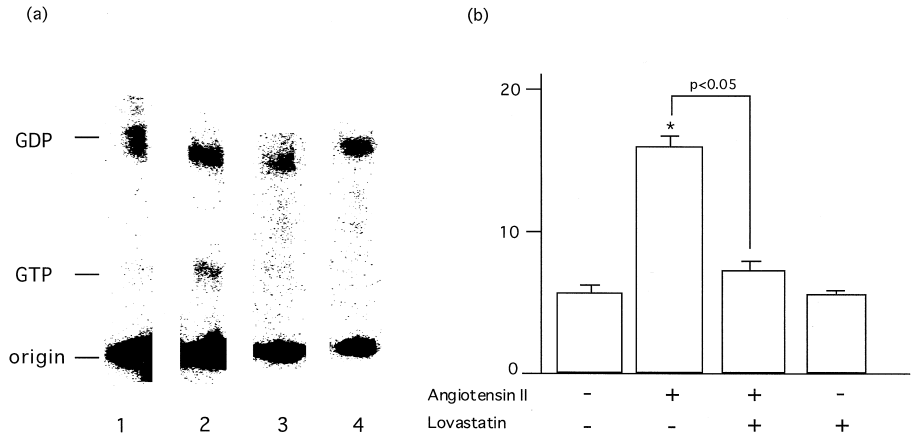

Fig. 3. Effects of angiotensin II and lovastatin on $p21^{ras}$ activation. Myocytes were labeled with [^{32}P]orthophosphate. Immunoprecipitation was performed by using an anti-v-H-ras antibody. Cardiac myocytes were stimulated with angiotensin II (10^{-6} M) for 10 min, and lovastatin (10^{-6} M) was added 24 h before exposure of angiotensin II. (a) Line 1: control cells, line 2: angiotensin II-treated cells, line 3: angiotensin II- and lovastatin-treated cells, line 4: lovastatin-treated cells. (b) Values are means ± S.E.M. of eight dishes in four different preparations and are ratios of GTP-bound $p21^{ras}$/GTP-bound $p21^{ras}$ + GDP-bound $p21^{ras}$. $^{*}P < 0.05$, compared with control cells at the same point.

3.4. Effects of angiotensin II, lovastatin and calphostin C on MAP kinase activity

Yamazaki et al. (1995) and our preliminary studies indicate that maximal activity of MAP kinase was reached at 8 min after exposure to angiotensin II. Therefore, the effects of angiotensin II, lovastatin and calphostin C on MAP kinase activity were evaluated at that time of exposure. Lovastatin (10^{-6} M), mevalonate (10^{-4} M) or calphostin C (10^{-6} M) was added 24 h, 24 h or 30 min before exposure, respectively. As shown in Fig. 2, angiotensin II significantly increased MAP kinase activity by 219% compared with 0 min. Lovastatin significantly inhibited angiotensin II-induced increases in MAP kinase activity by 37%, and this inhibitory effect of lovastatin was completely prevented in the presence of mevalonate (Fig. 2). On the other hand, calphostin C significantly inhibited angiotensin II-induced increases in MAP kinase activity by 80% (Fig. 2). Furthermore, pretreatment with both lovastatin and calphostin C completely inhibited the increases in MAP kinase activity induced by angiotensin II. Lovastatin or calphostin C alone did not change MAP kinase activity (Fig. 2).

3.5. Effects of angiotensin II and lovastatin on $p21^{ras}$ activation

The next experiment was undertaken to determine whether lovastatin inhibits the activation of $p21^{ras}$ induced by angiotensin II. Sadoshima and Izumo (1996) indicated that maximal activity of $p21^{ras}$ was reached at 10 min after exposure of angiotensin II. Therefore, the effects of angiotensin II (10^{-6} M) and lovastatin (10^{-6} M) on $p21^{ras}$ activity were evaluated at that time of exposure. As shown in Fig. 3a, basal levels of GTP-bound $p21^{ras}$ in untreated cardiac myocytes could not be clearly detected, and angiotensin II increased GTP-bound $p21^{ras}$. Angiotensin II significantly increased ratios of GTP-bound $p21^{ras}$ /GDP-bound $p21^{ras}$ + GTP-bound $p21^{ras}$ by 2.8-fold (from 5.9 ± 0.8 to 16.8 ± 1.3%, Fig. 3b). Lovastatin significantly inhibited the angiotensin II-induced increases in ratios of GTP-bound $p21^{ras}$/GDP-bound $p21^{ras}$ + GTP-bound $p21^{ras}$ by 85% (from 16.8 ± 1.3 to 7.6 ± 0.9%, Fig. 3b). Lovastatin alone did not change this ratios (Fig. 3b).

3.6. Effects of angiotensin II and lovastatin on $p21^{ras}$ membrane association

The next experiment was undertaken to determine if lovastatin inhibits the angiotensin II-induced conversion of cytoplasmic $p21^{ras}$ to membrane $p21^{ras}$. Cardiac myocytes were stimulated with angiotensin II (10^{-6} M) for 10 min, and lovastatin (10^{-6} M) was added 24 h before exposure of angiotensin II. As shown in Fig. 4, angiotensin II increased $p21^{ras}$ membrane association, and lovastatin inhibited it. Lovastatin alone did not change $p21^{ras}$ membrane association.

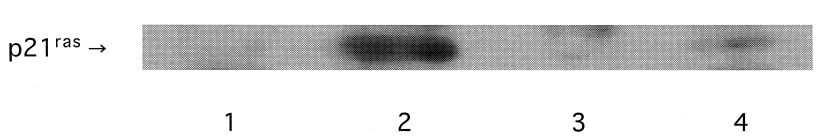

Fig. 4. Effects of angiotensin II and lovastatin on $p21^{ras}$ membrane association. Cardiac myocytes were stimulated with angiotensin II (10^{-6} M) for 10 min, and lovastatin (10^{-6} M) was added 24 h before exposure of angiotensin II. Line 1: control cells, line 2: angiotensin II-treated cells, line 3: angiotensin II- and lovastatin-treated cells, line 4: lovastatin-treated cells.

4. Discussion

Many diverse extracellular stimuli — including growth factors, hormones, osmolar shock, stress, and elevated temperature — results in activation of phosphorylation cascades utilizing MAP kinase (Bogoyevitch and Sugden, 1996). Stimulation of the p42 and p44 isoforms of MAP kinase (Erk2 and Erk1, respectively) requires dual phosphorylation on Thr-183 and Tyr-185 residues. A dual specificity protein kinase, MAP kinase–kinase (MEK), catalyzes the phosphorylation of MAP kinase and is itself regulated by serine phosphorization by MEK kinase and/or Raf kinase (Kyriakis et al., 1992; Crews et al., 1994). Angiotensin II stimulates hypertrophic growth of vascular smooth muscle cells and activates MAP kinase (Duff et al., 1992). Recently, Sadoshima and Izumo (1996) have reported that angiotensin II evokes the activation of MAP kinase in neonatal rat cardiac myocytes. The present experiments demonstrate that: (1) angiotensin II causes accelerated protein synthesis and cell hypertrophy, and that (2) angiotensin II activates MAP kinase. These data, combined with results previously presented, indicate that MAP kinase may be a critical intermediate in the signal transduction pathway of angiotensin II-induced hypertrophy.

Both protein kinase C-dependent and protein kinase C-independent regulatory events are required for Raf-1 activation leading to increased MAP kinase activity (Blenis, 1993). Protein kinase C directly phosphorylates and activates c-Raf, which leads to stimulation of MAP kinase pathway (Daum et al., 1990). We have already shown that angiotensin II activates protein kinase C through the type 1 angiotensin II receptor in cardiac myocytes (Miyata and Haneda, 1994). In the present study, we demonstrate that a selective protein kinase C inhibitor, calphostin C partially but significantly inhibits the angiotensin II-induced increases in MAP kinase activity and protein synthesis in cultured cardiomyocytes. These results suggest that protein kinase C may increase the activity of MAP kinase, which enhances protein synthesis in cardiac myocytes.

The protein kinase C-independent events leading to Raf-1 activation may involve $p21^{ras}$ (Bogoyevitch and Sugden, 1996). It has been shown that the active $p21^{ras}$ associates with other intracellular signaling molecules, such as Raf-1 and phosphatidyl-inositol-3 kinase, thereby regulating the downstream protein kinase cascades, including the MEK–MAP kinase–90 kDa S6 kinase (RSK) pathway (Blenis, 1993; Avrush et al., 1994; Rodriguez-Viciana et al., 1994). Activation of $p21^{ras}$ has been shown to occur after treatment of cells with a variety of stimuli, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin (Satoh et al., 1992). Thorburn et al. (1993) have reported that a $p21^{ras}$-dependent pathway, at least in part, mediates hypertrophy following stimulation of a classical G protein-coupled receptor in neonatal rat cardiac myocytes. Recently, Sadoshima and Izumo (1996) have reported that in cardiac myocytes angiotensin II induces $p21^{ras}$ activation, which requires tyrosine kinase activity, but does not require protein kinase C activation or an increase in intracellular Ca^{2+}. However, Zou et al. (1996) have reported that $p21^{ras}$ is not necessary in angiotensin II-induced activation of Raf/MAP kinase pathway in cardiac myocytes and that protein kinase C activation is critical.

Membrane localization of $p21^{ras}$, which is important for its vitro transforming activity, is normally dependent on post-translational modification of the carboxy terminus by sequential farnesylation, proteolysis, and carboxymethylation (Willumsen et al., 1984; Hancock et al., 1989). Since blocking post-translational farnesylation of $p21^{ras}$ precludes subsequent membrane localization, it could prevent the transforming potential of oncogenic $p21^{ras}$. On the other hand, mevalonate is synthesized intracellularly from HMG-CoA and this process is catalyzed by the enzyme HMG-CoA reductase. Therefore, the inhibition of the synthesis of farnesyl-1-diphosphate, by blocking the formation of the precursor mevalonate by HMG-CoA reductase inhibition, effectively prevents post-translational proceeding of Ras and subsequent membrane localization (Hancock et al., 1989; Maltese, 1990). Repko and Maltese (1989) have reported that a HMG-CoA reductase inhibitor, lovastatin, causes a rapid depletion of isoprenoid groups available for protein modification in Friend murine erythroleukemia cells and that mevalonate prevents the isoprenoid group depletion induced by lovastatin. O'Donnell et al. (1993) have reported that lovastatin inhibits serum-induced proliferation of rat mesangial cells and that its inhibitory effect is completely prevented in the presence of exogenous mevalonate. Recently, we have reported that lovastatin as well as calphostin C prevents stretch-induced cardiac hypertrophy in cultured neonatal rat heart cells (Kashiwagi et al., 1998). Therefore, we investigated the effects of lovastatin on the angiotensin II-induced hypertrophy of cultured neonatal rat heart myocytes to determine whether angiotensin II activates the $p21^{ras}$-dependent MAP kinase pathway or not. Lovastatin partially but significantly inhibited the angiotensin II-induced increases in MAP kinase activity and cell growth. This inhibitory effect of lovastatin on the angiotensin II-induced cell growth was completely prevented in the presence of mevalonate. Furthermore, pretreatment with both lovastatin and calphostin C completely inhibited the angiotensin II-induced increases in these parameters. As protein-to-DNA ratios were measured only at one time point in the present experiment, there are two alternative possibilities to explain the partial inhibition of angiotensin II-induced cell growth: (1) the inhibitors decreased steady state protein levels once cell growth had occurred, (2) the inhibitors caused a transient decrease in the rate of cell growth that was eventually overcome. Our previous data (Kashiwagi et al., 1998) has shown that pretreatment with lovastatin or calphostin C inhibits the stretch-induced increases in protein-to-DNA ratios on culture days 5, 6 and 7. Therefore, the inhibitors may de-

crease the rate of cell growth in angiotensin II-treated cells in the present experiment. These results suggest that angiotensin II may activate the $p21^{ras}$/MAP kinase pathway in cultured neonatal rat heart cells and that both protein kinase C-dependent MAP kinase pathway and $p21^{ras}$-dependent MAP kinase pathway may at least in part, contribute to the mechanism of angiotensin II-induced cardiac hypertrophy.

Sadoshima and Izumo (1993, 1996) have reported that angiotensin II rapidly activates $p21^{ras}$ in cultured neonatal rat cardiac myocytes. Therefore, in the next experiment we studied whether lovastatin directly inhibited $p21^{ras}$ activation induced by angiotensin II. Angiotensin II significantly enhanced the formation of the GTP-bound $p21^{ras}$ 2.8-fold in cultured rat heart cells, and lovastatin significantly inhibited the angiotensin II-induced increase in active $p21^{ras}$. Furthermore, angiotensin II increased the $p21^{ras}$ membrane association, and lovastatin inhibited it. These results strongly suggest that lovastatin may directly inhibits $p21^{ras}$ activation and the conversion of cytoplasmic $p21^{ras}$ to membrane $p21^{ras}$ induced by angiotensin II, which may partially prevent angiotensin II-induced increases in MAP kinases activity and cell growth.

There are differences in pharmacological lipophilicity, structure, solubility and action between HMG-CoA reductase inhibitors. Lipid-soluble HMG-CoA reductase inhibitors, as lovastatin, mevastatin and simvastatin, have methyl-substituents in the nucleus or in the ester side chain, while water-soluble HMG-CoA reductase inhibitors, as pravastatin, have a hydroxy-substituent in the nucleus (Serajuddin et al., 1991). Komai et al. (1992) have reported that both lovastatin and simvastatin are absorbed in rat spleen cells as well as rat liver cells, but that pravastatin is active only in rat liver cells in vitro. The results suggest that pravastatin is unable to pass though the plasma membrane of extrahepatic cells because of its hydrophilicity and the lack of a carrier system. Also, there is a difference between lipid-soluble HMG-CoA reductase inhibitors and water-soluble HMG-CoA reductase inhibitors in their potency to inhibit mevalonate synthesis in extrahepatic cells (Vliet et al., 1995). Corsini et al. (1991) have reported that simvastatin inhibits the proliferation of myocytes from the rat aorta, but pravastatin does not. Therefore, in the present study we compared the effect of lovastatin on angiotensin II-induced cardiac hypertrophy with those of simvastatin and pravastatin. Simvastatin as well as lovastatin had an inhibitory effect on angiotensin II-stimulated cell growth, but pravastatin did not. These results suggest that HMG-CoA reductase inhibitors may have the different effects on cardiac hypertrophy that depend, at least in part, on their chemical properties.

In summary, we have demonstrated that: (1) angiotensin II causes accelerated protein synthesis and cell hypertrophy in cultured neonatal rat heart cells; (2) lovastatin and simvastatin, but not pravastatin, partially prevented the angiotensin II-induced increases in protein synthesis and cell growth; (3) mevalonate overcame the inhibitory effects of lovastatin and simvastatin on angiotensin II-induced increases in these parameters; (4) calphostin C partially prevented the angiotensin II-induced increases in protein synthesis and cell growth, and treatment with both lovastatin and calphostin C resulted in complete inhibition; (5) lovastatin or calphostin C partially prevented the angiotensin II-induced increases in MAP kinase activity, and treatment with both lovastatin and calphostin C inhibited completely; (6) angiotensin II increased $p21^{ras}$ activity and the conversion of cytoplasmic $p21^{ras}$ to membrane $p21^{ras}$, and lovastatin prevented them. These studies suggest that a HMG-CoA reductase inhibitor, lovastatin, may prevent angiotensin II-stimulated cardiac hypertrophy through $p21^{ras}$/MAP kinase pathway, which is linked to mevalonate metabolism. Further research is required to investigate the cross-talk between these signal transduction pathways so that the spatial and temporal relationships that integrate the multiple signaling events and lead to adaptational growth of the ventricular myocyte may be understood.

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