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Preventive effects of bicarbonate on cerivastatin-induced apoptosis

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Abstract

Although HMG-CoA reductase inhibitors such as statins are the most widely used cholesterol-lowering agents, there is a risk of myopathy or rhabdmyolysis occurring in patients taking these drugs. It has been reported that a number of lipophilic statins cause apoptosis in various cells, but it is still not clear whether intracellular acidification is involved in statin-induced apoptosis. There have been few studies aimed at identifying compounds that suppress statin-induced myotoxicity. In the present study, we examined the relationship between cerivastatin-induced apoptosis and intracellular acidification and the effect of bicarbonate on cerivastatin-induced apoptosis using an RD cell line as a model of in vitro skeletal muscle. Cerivastatin reduced the number of viable cells and caused dramatic morphological changes and DNA fragmentation in a concentration-dependent manner. Moreover, cerivastatin-induced apoptosis was associated with intracellular acidification and caspase-9 and -3/7 activation. On the other hand, bicarbonate suppressed cerivastatin-induced pH alteration, caspase activation, morphological change and reduction of cell viability. Accordingly, bicarbonate suppressed statin-induced apoptosis. The strategy to combine statins with bicarbonate can lead to reduction in the chance of the severe adverse events including myopathy or rhabdmyolysis.

Keywords: Statins; Bicarbonate; Myopathy; Rhabdmyolysis; Acidification

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors such as statins are the most widely used cholesterol-lowering agents for prevention of obstructive cardiovascular events (Downs et al., 1998; Havel and Rapaport, 1995; Jukema et al., 1995). However, severe adverse events, including myopathy and rhabdmyolysis, associated with lipophilic statins sometimes limit the lipid-lowering therapy with these agents (Hodel, 2002; Thompson et al., 2003). It has been reported that a number of lipophilic statins cause apoptosis in various cells (Guijarro et al., 1999; Newton et al., 2002; Ogata et al., 2002), but there have been few studies aimed at identifying compounds that suppress statin-induced apoptosis.

Apoptosis, programmed cell death, is a unique physiological process of cell death that is essential for morphogenesis, tissue homeostasis, and host defence (Ellis et al., 1991). It is known that generation of artificial cytosolic acidification induces caspase activation and apoptosis (Baumgart et al., 1996; Hirpara et al., 2001; Juel, 1996). However, it is still not clear whether intracellular acidification is involved in statin-induced apoptosis.

Cerivastatin, one of statins, was used as an effective therapeutic agent (Moghadasian, 1999). However, this drug was voluntarily withdrawn from the market in 2001 due to a severe side effect, myotoxicity, which sometimes caused death (Charatan, 2001). The *n*-octanol/water partition coefficient of cerivastatin has been reported to be 2.32, indicating that it is lipophilic (Matsuyama et al., 2002). On the other hand, rosuvastatin is known to be a hydrophilic statin. However, there has been little investigation of rosuvastatin-induced cytotoxicity and the reasons for the difference between cytotoxicity of lipophilic statins and that of hydrophilic statins.

The aim of this study was to elucidate the mechanisms underlying the cytotoxicity of cerivastatin and rosuvastatin and to clarify the relationship between cerivastatin-induced apoptosis and intracellular acidification. Since bicarbonate is clinically used for treatment of lactic acidosis (Kawanishi et al., 1998) and is involved in cellular pH regulation (alkalization) (Izumi

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et al., 2003), the effect of bicarbonate on cerivastatin-induced apoptosis was investigated.

2. Materials and methods

2.1. Chemicals

Cerivastatin Na and rosuvastatin Ca were kindly donated by Sankyo (Tokyo, Japan). All other reagents were of the highest grade available and used without further purification.

2.2. Cell culture

All experiments were carried out using cultured human rhabdomyosarcoma cells (RD cells) of the spindle-cell type obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). The RD cells, prototypic embryonal rhabdomyosarcoma cell line, is a tumor of skeletal muscle origin affecting children and young adults and expresses a number of muscle-specific proteins (Knudsen et al., 1998) and has been used as a model in which to study myotoxicity effects of statins (Nishimoto et al., 2003).

RD cells were maintained in plastic culture flasks (Corning Incorporated Corning) as described previously (Kobayashi et al., 2005). The RD cells were kept in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin-streptomycin at 37 °C under 5% CO_2.

2.3. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay was performed as described previously (Mosmann, 1983) with a certain modification. The MTT is normally reduced by dehydrogenases of viable cells and transformed to formazan. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. For the MTT assay, RD cells were seeded at density of 5–10 × 10^4 cells/mL on 96-well plastic plates. Following cell attachment (24 h), various concentrations of cerivastatin and rosuvastatin in combination with or without bicarbonate were added for the times indicated. At 4 h before the end of treatment, 10 μL of PBS-containing MTT solution (0.5%) was added, and the cells were incubated for a further 4 h. The MTT medium was then replaced with 0.2 ml dimetylsulfoxide, and absorbance was read at 590 nm. Absorbance measured in MTT assays was expressed as percent of the control (defined as 100%).

2.4. DNA fragmentation assay

Cells were scraped and centrifuged at 3000 rpm for 5 min and washed two times with 0.5 mL PBS. After 0.5 mL lysis buffer containing 10 mM EDTA, 200 mM NaCl, 0.1 mg/mL proteinase K, 0.2% TritonX-100 and Tris–HCl (pH 7.4) was added, PCI was added and suspended. The suspension was centrifuged at 12,000 rpm for 10 min, and the DNA in the supernatant was ethanol-precipitated. The sample was resuspended in 0.5 mL TE buffer containing 10 mM Tris and 1 mM EDTA and digested with RNase. After PCI had been added and suspended, the suspension was centrifuged at 12,000 rpm for 10 min, and the DNA in the supernatant was ethanol-precipitated. The sample was analyzed on a 2.0% agarose gel.

2.5. Uptake experiments

In the experiments on uptake of cerivastatin and rosuvastatin, after removal of the growth medium, cells were washed with HEPES buffer (25 mM D-glucose, 137 mM NaCl, 5.37 mM KCl, 0.3 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 10 mM HEPES, pH 7.4) and preincubated at 37 °C for 10 min with 0.5 mL of HEPES buffer (pH 7.4). Uptake was initiated by applying HEPES buffer (pH 7.4) containing 100 μM cerivastatin and rosuvastatin. The uptake study was performed at 37 °C. After a predetermined time period, uptake was terminated by suctioning off the applied solution and immersing the plates in ice-cold HEPES buffer (pH 7.4) and then suspended in 0.5 mL of ice-cold water. Samples were frozen at −80 °C until the assay. The cellular protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Cerivastatin and rosuvastatin were determined using an HPLC system equipped with a UV detector. The columns were YMC packed column A-312 ODS (5 μm, 150 mm × 6 mm i.d.) and Inertsil ODS-2 (5 μm, 150 mm × 4.6 mm i.d), and a mobile phase containing of 2.5 mM CH_3COONH_4:CH_3OH (1: 4, v/v) and 2.5 mM CH_3COONH_4:CH_3CN (1:1, v/v) was used. Column temperature and flow rate were 40 °C and 1.0 ml/min, respectively.

2.6. Measurement of intracellular pH

Intacellular pH was measured as described previously (Boyarsky et al., 1988) with a certain modification. Cells were harvested and resuspended in 0.5 mL of buffer (pH 7.4) containing 5 mM D-glucose, 20 mM HEPES, 5 mM KCl and 153 mM NaCl. The suspended cells were labeled at 37 °C for 30 min with 3 μM BCECF-AM. After removal of the buffer (pH 7.4) containing BCECF-AM, cells were washed two times. Intracellular BCECF-AM was excited at 485 and 450 nm and emission was analyzed at 535 nm. The ratio of BCECF-AM fluorescence was used to obtain cytosolic pH from a pH calibration curve. In order to make a pH calibration curve, the BCECF-AM-loaded cells described above were adjusted in a calibration buffer containing 10 mM NaCl, 1 mM $MgSO_4$, 10 mM Na-MOPS and 130 mM KCl ranging in pH from 8.0 to 6.2, and pH was held constant by incubation for 10 min in 10 μg/mL of the proton ionophore nigericin.

2.7. Caspase assay

Cells were lysed with a cell culture lysis reagent (Promega). Protein concentration of the cell lysate was adjusted to 10 μg/mL, and the cell lysate was assayed for caspase-3/7 and caspase-9 colorimetric protease assays measuring Ac-DEVD-

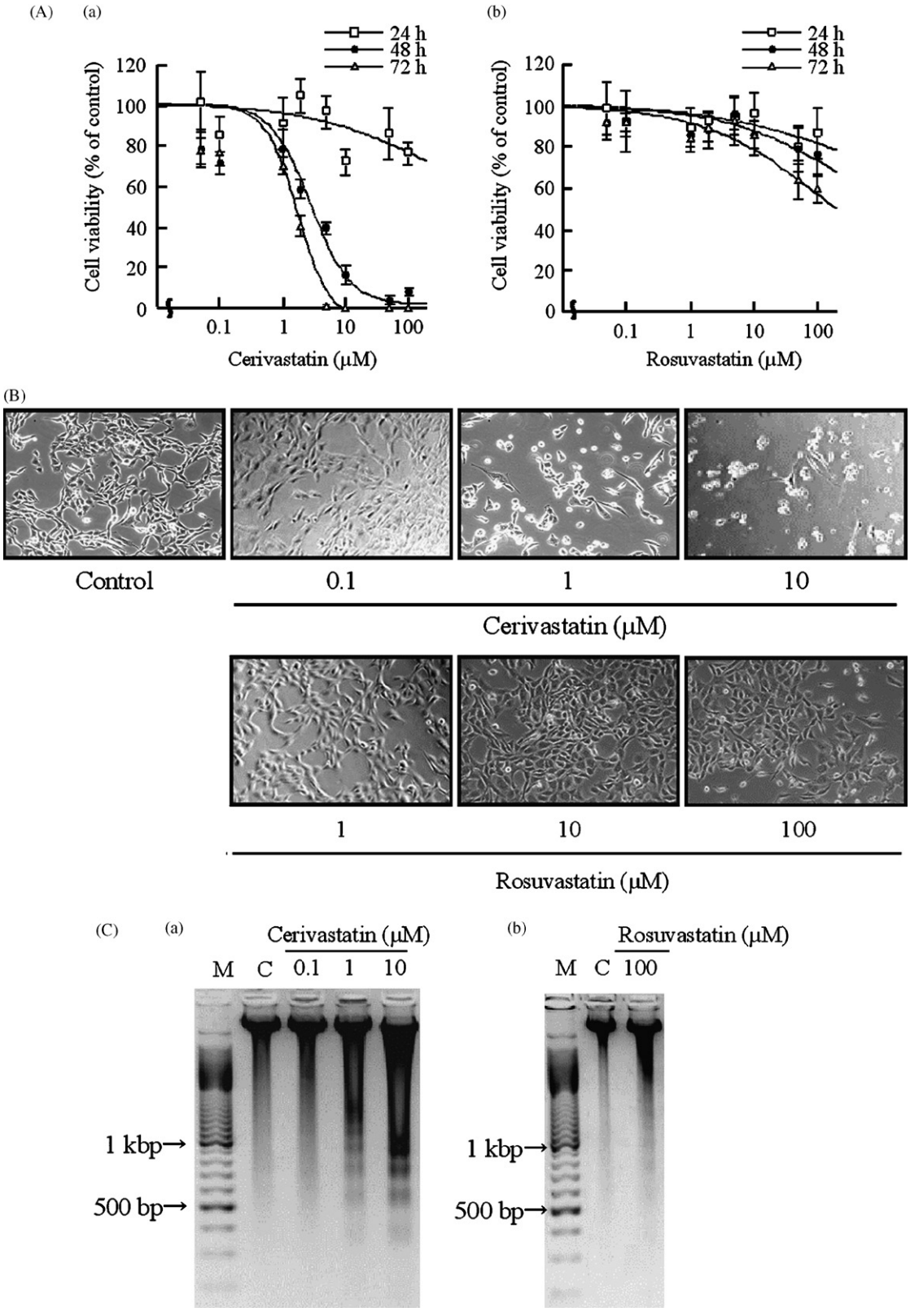

Fig. 1. (A) Effects of cerivastatin (a) and rosuvastatin (b) on the viability of RD cells. Cell viability was measured by the MTT assay. RD cells were exposed to various concentrations of cerivastatin and rosuvastatin for 24–72 h. Each point represents the mean ± S.D. of six determinations. (B) Morphological degeneration induced by cerivastatin and rosuvastatin. RD cells were exposed to various concentrations of cerivastatin and rosuvastatin for 48 h, and the cells were examined by microscopy (100×). (C) DNA fragmentation induced by cerivastatin (a) and rosuvastatin (b). RD cells were exposed to cerivastatin (0.1, 1 and 10 μM) and rosuvastatin (100 μM) for 48 h. Total DNA was extracted from cerivastatin and rosuvastatin-treated cells. Extracted DNA (20 μg) was electrophoresed on a 2% agarose gel. M: marker; C: control.

pNA and Ac-LEHD-pNA cleavage (Promega) as described in the manufacturer's protocol (Promega).

2.8. Analytical procedures

Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA). Statistical significance was defined as $P < 0.05$. The IC_{50} value was determined from the dose–response curve generated by using Origin® (version 6.1J).

3. Results

3.1. Cytotoxicity of cerivastatin and rosuvastatin in RD cells, prototypic embryonal rhabdomyosarcoma cell line

Firstly, to clarify the mechanisms of the cytotoxicity of statins, we performed MTT assay and examined the effects of cerivastatin, a lipophilic statin, and rosuvastatin, a hydrophilic statin, on RD cell viability. As shown in Fig. 1(A), cerivastatin reduced a number of viable cells in time- and concentration-dependent manners. In contrast, rosuvastatin had little influence on cell viability. Based on these data, we calculated the IC_{50} values for cerivastatin to be 1.77 μM. Next, in order to determine the mechanisms of the cytotoxicity of statins, we examined the morphological effects of cerivastatin and rosuvastatin on RD cells. As shown in Fig. 1(B), RD cells underwent dramatic morphological changes in a concentration-dependent manner after exposure to cerivastatin for 48 h. In contrast, rosuvastatin had no effect on cell morphology. Moreover, as shown in Fig. 1(C), cerivastatin caused DNA fragmentation in a concentration-dependent manner as assessed by DNA electrophoresis, whereas rosuvastatin had no effect. Accordingly, a lipophilic statin cytotoxicity is greater than that of a hydrophilic statin.

3.2. Accumulation of cerivastatin and rosuvastatin in RD cells

To clarify the difference between the cytotoxicity of cerivastatin and that of rosuvastatin, we examined the accumulation of cerivastatin and rosuvastatin in RD cells. The time courses of accumulation of cerivastatin and rosuvastatin reached an equilibrium in 30 min, and cerivastatin accumulation was about 10-fold greater than that of rosuvastatin at each time (Fig. 2). These results suggest that statin-induced cytotoxicity is associated with intracellular accumulation of statins.

In the following study, we focused on the mechanism of cerivastatin-induced cytotoxicity.

3.3. Mechanism of the cytotoxicity of cerivastatin on RD cells

To clarify the mechanism of the cyototoxicity of cerivastatin, we examined the activation of caspases. The effector caspase-3/7 plays a central role in apoptosis since it translocates from the cytosol into the nucleus upon activation (Ferri and Kroemer, 2001). As shown in Fig. 3(A), caspase-3/7 activity showed a strong activation peak for 48 h upon cerivastatin exposure and cerivastatin markedly enhanced the activity of caspase-3/7 in a concentration-dependent manner. As shown in Fig. 3(B), caspase-9 activity was elevated in a concentration-dependent manner after exposure to cerivastatin for 48 h. Moreover, the activation of caspase-3/7 and caspase-9 were blocked by caspase-9 inhibitor zLEHD-fmk (Fig. 3(C)). Accordingly, cerivastatin mainly induced apoptosis via a mitochondrial stress-induced cascade in RD cells.

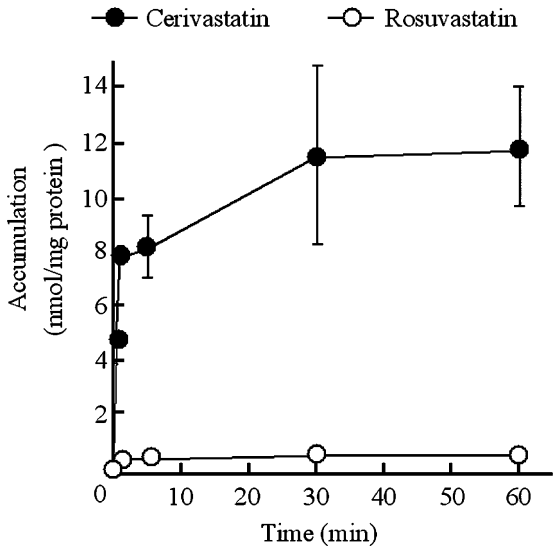

Fig. 2. Accumulation of cerivastatin and rosuvastatin in RD cells. The accumulation of cerivastatin and rosuvastatin (100 μM) in RD cells was measured at pH 7.4. Each point represents the mean ± S.D. of three determinations.

It is known that generation of artificial cytosolic acidification induces caspase activation and apoptosis (Baumgart et al., 1996; Hirpara et al., 2001; Juel, 1996). To elucidate the relationship between cerivastatin-induced apoptosis and intracellular acidification, we examined intracellular pH after exposure to cerivastatin for 48 h. As shown in Fig. 3(D), intracellular pH markedly decreased in a concentration-dependent manner. Moreover, intracellular acidification induced by cerivastatin was distinguished at a concentration of more than 1 μM.

3.4. Preventive effects of bicarbonate on cerivastatin-induced apoptosis of RD cells

Since cerivastatin-induced apoptosis was associated with intracellular acidification (Fig. 3(D)), we speculated that cerivastatin-induced cytotoxicity was suppressed by alkalization. Bicarbonate is clinically used for treatment of lactic acidosis (Kawanishi et al., 1998) and is involved in cellular pH regulation (alkalization) (Izumi et al., 2003). We therefore examined the effects of a combination of cerivastatin and bicarbonate on intracellular pH. As shown in Fig. 4(A), bicarbonate significantly inhibited cerivastatin-induced intracellular acidification. Moreover, Fig. 4(B) and (C) shows that the combination of cerivastatin and bicarbonate also prevented morphological change and caspase-3/7 activation by cerivastatin

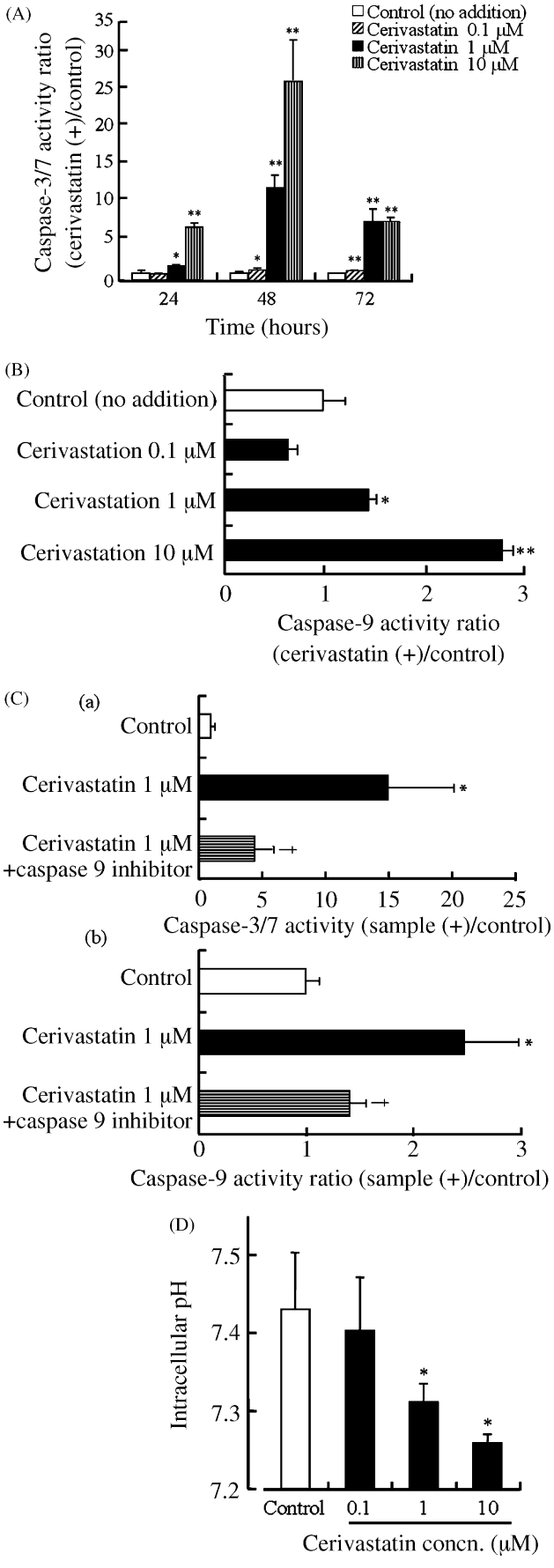

Table 1
Effect of sodium bicarbonate on growth inhibition of cerivastatin

	IC_{50} value (μM)	Ratio
Cerivastatin	1.77	
Cerivastatin + $NaHCO_3$	6.27	3.54

RD cells were exposed to various concentrations of cerivastatin in the absence or presence of 25 mM sodium bicarbonate (pH 7.4) for 48 h. The conditions were identical to those described in the legend to Fig. 1(A). IC_{50} values were estimated by using Origin® (version 6.1J). Each value represents the mean ± S.D. of six-twelve determinations. Ratio: (cerivastatin + sodium bicarbonate)/(cerivastatin).

alone. Accordingly, bicarbonate suppresses cerivastatin-induced apoptosis. Table 1 shows the effects of bicarbonate on the viability of RD cells treated with cerivastatin. Bicarbonate suppressed cerivastatin-induced reduction in cell number. We calculated the IC_{50} values for cerivastatin and the combination of cerivastatin and bicarbonate to be 1.77 and 6.27, respectively.

4. Discussion

In terms of the more safety use of statins, the mechanism of statin-induced cytotoxicity must be clarified. Firstly, to confirm the mechanism of cerivastatin-induced apoptosis in RD cells, *in vitro* skeletal muscle model, we examined the effects of cerivastatin and rosuvastatin on viability, morphology and DNA degradation of RD cells. Cerivastatin reduced the number of viable cells and caused dramatic morphological changes and DNA fragmentation in a concentration-dependent manner (Fig. 1). Cerivastatin-induced cyototoxicity is associated with apoptosis. In contrast, rosuvastatin had no effect on viability, morphology or DNA degradation of RD cells (Fig. 1). To determine whether there is a correlation between the potency of cytotoxicity of statins and their cellular incorporation, we examined the accumulation of cerivastatin and rosuvastatin in RD cells. Cerivastatin accumulation was about 10-fold greater than that of rosuvastatin at each time (Fig. 2). This result suggests that statin-induced cytotoxicity is associated with intracellular accumulation of statins.

Fig. 3. (A) Effect of cerivastatin on caspase-3/7 activity ratio in RD cells. RD cells were exposed to various concentrations of cerivastatin for 24–72 h, and the cell lysate was used to determine the caspase-3/7 activity ratio. Each column represents the mean with S.D. of three determinations. *Significantly different from control (no addition) at $p < 0.05$, **$p < 0.01$. (B) Effect of cerivastatin on caspase-9 activity ratio in RD cells. RD cells were exposed to various concentrations of cerivastatin for 48 hours, and the cell lysate was used to determine the caspase-9 activity ratio. Each column represents the mean with S.D. of three determinations. *Significantly different from control (no addition) at $p < 0.05$; **$p < 0.01$. (C) Effect of caspase-9 inhibitor on caspase-3/7 (a) and -9 (b) activity ratio in cerivastatin-treated RD cells. RD cells were exposed to cerivastatin (1 μM) in the absence or presence of 10 μM caspase-9 inhibitor for 48 h. The conditions were identical to those described in the legend to (A). Each column represents the mean with S.D. of three determinations. *Significantly different from control (no addition) at $p < 0.01$, †significantly different from cerivastatin alone at $p < 0.05$. (D) Effect of cerivastatin on pH in RD cells. Intracellular acidification was measured with BCECF-AM at 48 h as described in Section 2. Each column represents the mean with S.D. of three-six determinations. *Significantly different from control (no addition) at $p < 0.05$.

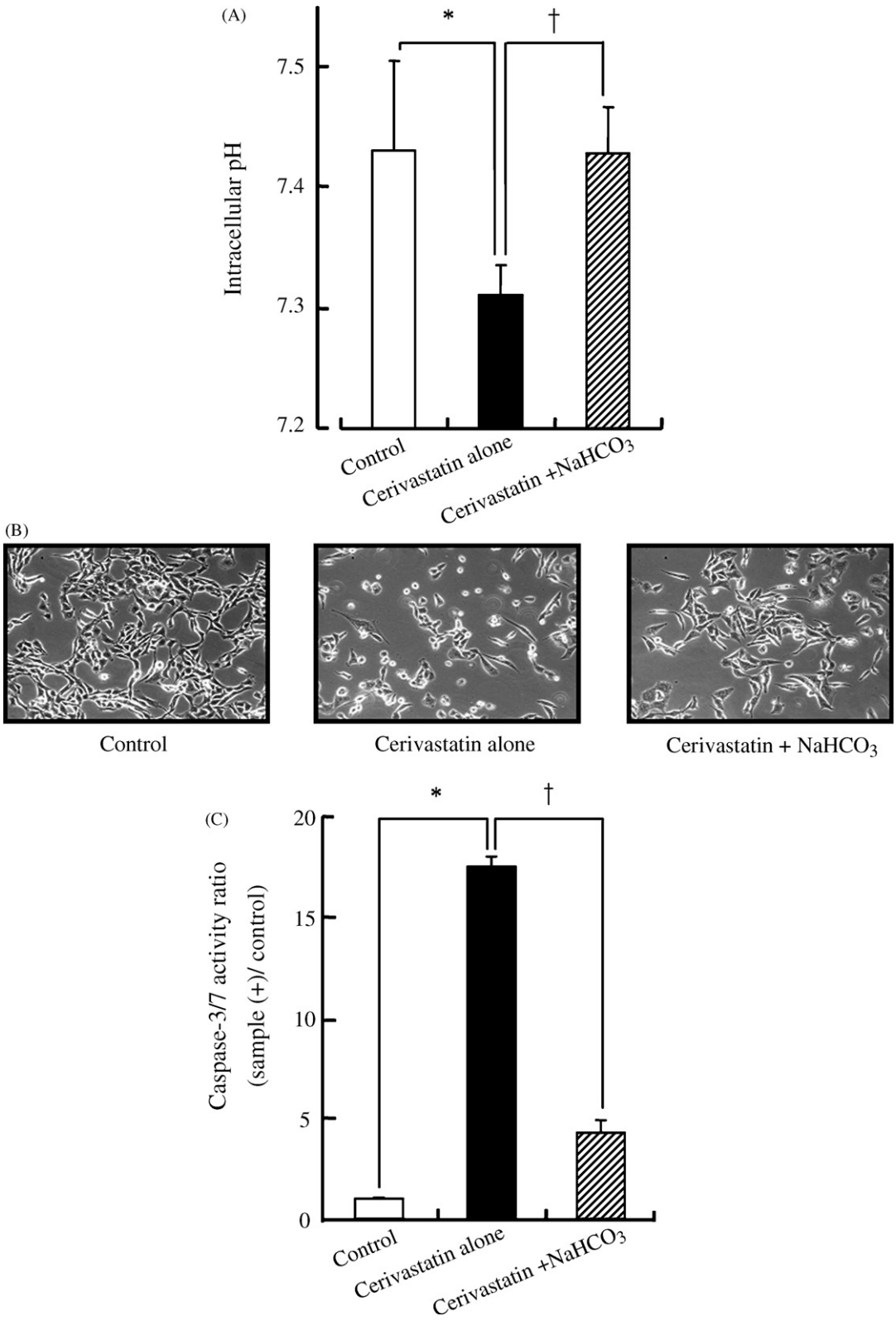

Fig. 4. (A) Effect of sodium bicarbonate on acidification in cerivastatin-treated RD cells. RD cells were exposed to cerivastatin (1 μM) in the absence or presence of 25 mM sodium bicarbonate (pH 7.4) for 48 h. The conditions were identical to those described in the legend to Fig. 3(D). Each column represents the mean with S.D. of three-six determinations. *Significantly different from control (no addition) at $p < 0.05$, †significantly different from cerivastatin alone at $p < 0.05$. (B) Effect of sodium bicarbonate on morphological degeneration in cerivastatin-treated RD cells. RD cells were exposed to cerivastatin (1 μM) in the absence or presence of 25 mM sodium bicarbonate (pH 7.4) for 48 h. The conditions were identical to those described in the legend to Fig. 1(B). (C) Effect of sodium bicarbonate on caspase-3/7 activity ratio in cerivastatin-treated RD cells. RD cells were exposed to cerivastatin (1 μM) in the absence or presence of 25 mM sodium bicarbonate (pH 7.4) for 48 h. The conditions were identical to those described in the legend to Fig. 3(A). Each column represents the mean with S.D. of three determinations. *Significantly different from control (no addition) at $p < 0.01$, †significantly different from cerivastatin alone at $p < 0.05$.

Next, we focused on the molecular mechanism leading to apoptosis by cerivastatin in RD cells. It has been demonstrated that caspases are important mediators of apoptosis. Various apoptotic stimulation activate the machinery; caspase-9 is activated by cytochrome *c* released from mitochondria. The activated caspase-9 converts inactive pro-caspase-3 into active caspase-3, resulting in chromosomal DNA fragmentation and cellular morphologic changes characteristic of apoptosis (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Caspase-9 and -3/7 were activated in a concentration-dependent manner (Fig. 3(A) and (B)). The activation of caspase-9 and -3/7 was blocked by caspase-9 inhibitor zLEHD-fmk (Fig. 3(C)). Accordingly, we suggest that cerivastatin mainly induced apoptosis via a mitochondrial stress-induced cascade in RD cells. To elucidate the upstream of cerivastatin-induced apoptosis pathway, we examined the relationship between cerivastatin-induced apoptosis and intracellular acidification. It was previously shown that cytosolic acidification is important in caspase activation (Hirpara et al., 2001; Matsuyama et al., 2000) and caused via mitochondrial stress-induced cascade (Jeong et al., 2001). Cerivastatin-induced intracellular acidification in a concentration-dependent manner (Fig. 3(D)). Moreover, the intracellular acidification was not blocked by caspase-9 inhibitor zLEHD-fmk (data not shown). After cerivastatin-induced intracellular acidification, activated caspase-9 and -3/7 in RD cells. Now, we try to clarify the mechanism of cerivastatin-induced intracellular acidification. We previously reported that L-lactic acid efflux is mediated by MCT4 in RD cells (Kobayashi et al., 2005). The efflux of L-lactic acid from RD cells was significantly inhibited by lipophilic statins. On the other hand, hydrophilic statins had no effect on the efflux of L-lactic acid from RD cells (data not shown). Moreover, we established a CD147 and MCT4 co-transfected cell line (cm) and examined the inhibitory effects of statins on L-lactic acid uptake by cm. Lipophilic statins significantly inhibited L-lactic acid uptake concentration dependently. On the other hand, the inhibitory effects of hydrophilic statins were very weak (Kobayashi et al., 2006). Accordingly, inhibitory effect of statins on L-lactic acid transport mediated by MCT4 can suggest the risk of leading to over-accumulate lactic acid and being not to maintain muscle homeostasis, moreover, the possibility of inducing intracellular acidification and apoptosis in skeletal muscle.

To avoid severe adverse events, including myopathy and rhabdmyolysis, it is necessary to investigate the compound suppressing statin-induced apoptosis. Bicarbonate is clinically used for treatment of lactic acidosis (Kawanishi et al., 1998) and is involved in cellular pH regulation (alkalization) (Izumi et al., 2003). Since cerivastatin-induced apoptosis was associated with intracellular acidification, we therefore examined bicarbonate-induced improvement of statin-induced apoptosis. Bicarbonate suppressed the reduction of cell viability induced by cerivastatin in a concentration-dependent manner within 25 mM (data not shown). Accordingly, we selected subsequently bicarbonate concentration was 25 mM. Bicarbonate significantly inhibited cerivastatin-induced caspase-3/7 activation (Fig. 4(C)). Moreover, Fig. 4(A) and (B) shows that the combination of cerivastatin and bicarbonate also prevented intracellular acidification and cell shrinkage by cerivastatin alone. Cell shrinkage is a hallmark in apoptosis has been shown to be a signal in itself for the apoptotic process (Okada et al., 2001; Friis et al., 2005). When bicarbonate is present, Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger will result in a regulatory volume increase resulting from uptake of Na^+ and Cl^-. Since the cerivastatin-induced cell shrinkage is an important signal to apoptosis, this regulatory volume increase will protect against apoptosis. On the other hand, in nominally absence of HCO_3^-, activation of Na^+/H^+ exchanger will result in a partly reestablishment of a normal pH without in cell volume (Hoffmann and Dunham, 1995). Further investigations to clarify whether the protective effect of bicarbonate is caused by the fact that cells can regain their volume when bicarbonate is present by the coupled effect of the Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger are in progress.

Interestingly, although the mechanism of statin-induced cytotoxicity has been investigated (Guijarro et al., 1999; Newton et al., 2002; Ogata et al., 2002), there have been few investigations on compounds suppressing statin-induced cytotoxicity.

Further investigations to obtain the result that bicarbonate-induced improvement of statin-induced tissue damage from in vivo study are in progress.

In the present study, we showed that statin-induced cytotoxicity is associated with intracellular accumulation of statins and that cerivastatin-induced apoptosis was associated with their intracellular acidification. Furthermore, the combination of cerivastatin and bicarbonate suppressed apoptosis of RD cells by decreasing caspase activation and preventing morphological change.

Thus, the strategy to combine statins with bicarbonate may lead to reduce the chance of the severe adverse events including myopathy, rhabdmyolysis. Finally, this approach may prove to be inexpensive, safe, and useful to increase the efficacy of statins in hyperlipidemia patient therapy.

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