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FEBS Letters 343 (1994) 151–154

FEBS 13918

FEBS LETTERS

Simvastatin-sodium delays cell death of anoxic cardiomyocytes by inhibition of the Na^+/Ca^{2+} exchanger

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Received 24 February 1994

Abstract

When incubated under anoxic conditions, cultured neonatal cardiomyocytes undergo cell necrosis. Simvastatin-sodium, the bioactive metabolite of simvastatin (a potent serum cholesterol-lowering drug), delayed the anoxia-induced myocyte necrosis in a dose-dependent manner. This beneficial effect of simvastatin-sodium could not be attributed to its cholesterol-lowering properties. We found that simvastatin-sodium, at concentrations of 20 and 50 μM, attenuated the rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measured with Fura-2 in anoxic cardiomyocytes. In a test of sarcolemmal Na^+/Ca^{2+} exchange activity, simvastatin-sodium attenuated the rise of $[Ca^{2+}]_i$ upon incubation in sodium-free buffer, which normally causes a reversal of Na^+/Ca^{2+} exchange and cellular calcium overload. The inhibitory action of simvastatin-sodium on the sarcolemmal Na^+/Ca^{2+} exchanger could well explain the cardioprotective effect of the drug on myocytes subjected to anoxia.

Key words: Simvastatin-sodium; Na^+/Ca^{2+}-exchanger; Intracellular $[Ca^{2+}]$; Anoxia; Cell death

1. Introduction

Simvastatin-sodium, the bioactive metabolite of simvastatin, is an inhibitor of the rate-determining enzyme in the biosynthesis of cholesterol, β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA) reductase, and is used as an anti-hyperlipidemic drug in humans [1,2]. In addition, inhibitors of HMG-CoA reductase have been used experimentally to lower cellular cholesterol content of cultured cells [3].

Earlier, we reported that modulation of cellular cholesterol content of cultured cardiomyocytes using liposomes altered the tolerance to anoxia [4,5]. Recently, we employed simvastatin-sodium in an attempt to diminish cellular cholesterol content. We found that simvastatin-sodium had a protective effect on cardiomyocytes during anoxia that could not be attributed to its cholesterol-lowering capabilities. The simvastatin-sodium-induced delay of cell death during anoxia was associated with an attenuation of the rise of the intracellular Ca^{2+} concentration in cardiomyocytes. To elucidate the mechanism responsible for the beneficial effects of simvastatin-sodium on anoxic myocytes, we investigated whether simvastatin-sodium affects the Na^+/Ca^{2+}-exchanger. The Na^+/Ca^{2+} exchanger is considered to be responsible, at least in part, for intracellular Ca^{2+} overload during anoxia [6,7].

2. Material and methods

2.1. Neonatal cardiomyocytes

Two-day-old rats were anesthetized with diethylether. Their hearts were excised and the ventricles dissociated using collagenase as the dissociating enzyme [8]. Cultures were grown for 3 days in a culture medium containing Ham's F-10 (Flow) with 10% fetal calf serum (Flow) and 10% horse serum (Flow) on plastic Petri dishes (diameter 30 mm, primaria coated; Falcon) and maintained at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Culture medium was refreshed after 4 h and after 48 h. At the third day after plating the cells had formed a monolayer and were used for the experiments. Intracellular calcium ion concentration ($[Ca^{2+}]_i$) was measured in cultures grown on glass coverslips (10 × 32 mm) coated with laminin (Gibco) in a plastic Petri dish.

2.2. Simvastatin-sodium

The bioactive metabolite of simvastatin (Zocor; Merck, Sharp & Dohme) was formed in vitro by heating (60°C) a 1 mM solution of simvastatin in 0.1 N NaOH for 1 h [9]. The resulting 1 mM solution of simvastatin-sodium was brought to pH 7.5 with 12 N HCl.

2.3. Anoxic incubation

Cultures were preincubated in a balanced salt solution (BSS) which contained (in mM): NaCl 140, KCl 4.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 0.44, Na_2HPO_4 0.34, $NaHCO_3$ 21; sodium pyruvate 5; pH 7.4, for 1 h (37°C, 95% air/5% CO_2). Then the medium was poured off and replaced by BSS equilibrated for at least 1 h with 95% N_2/5% CO_2 (resulting in a $pO_2 < 5$ mmHg). Eight dishes at a time were placed in an anoxic incubation chamber with a continuous gas flow of 95% N_2/5% CO_2 at 37°C.

2.4. Cell death

At indicated time points during the anoxic incubations, aliquots of the medium were taken to measure lactate dehydrogenase (LDH) activity released by the cells. After anoxic incubation the medium was removed, and the cardiomyocytes were taken up in 1.0 ml ice-cold Tris buffer (10 mM, pH 7.4). The cells were homogenized in a glass Potter–Elvehjem homogenizer and then sonicated for 1 min at 30 W (Branson Sonic Power Co.). Medium and cellular homogenate were analyzed for LDH activity using a spectrophotometric assay (Boehringer 543047).

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SSDI 0014-5793(94)00303-D

The release of LDH during metabolic inhibition was expressed as the percentage of total (pre-anoxic) cellular LDH.

2.5. $[Ca^{2+}]_i$ measurement

Confluent myocyte cultures grown on glass coverslips were rinsed with a HEPES-buffered salt solution (HBSS) containing (in mM): NaCl 125, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1, KH_2PO_4 1, $NaHCO_3$ 10, HEPES 10, glucose 5, probenecid 2.5; pH 7.4. The cells were loaded in HBSS containing 2 μM Fura-2/AM (Molecular Probes) for 60 min at 37°C [10]. Subsequently, the coverslips with the cells were washed twice in HBSS and fitted in a cuvette in a spectrofluorometer (Perkin-Elmer LS-3) equipped with a thermostated cuvette holder (37°C). The fluorescence of Fura-2-loaded cells was measured at 340 and 380 nm excitation wavelengths and 490 nm emission wavelength. The ratio of the fluorescence intensities measured at excitation wavelengths 340 nm and 380 nm, F_{340}/F_{380}, provides an index for $[Ca^{2+}]_i$ [11]. As the numerous problems concerning the calibration of Fura-2 have not been resolved, we have chosen not to express the ratio values in nM Ca^{2+} concentration. Instead, the F_{340}/F_{380} ratio values during the experiments were expressed as a percentage increase compared to the control value at the start of the experiment.

2.6. Na^+/Ca^{2+} exchanger

The activity of the Na^+/Ca^{2+} exchanger was measured by incubating the cardiomyocytes in Na^+-free HBSS. In this medium, sodium salts were replaced by equimolar quantities of choline chloride. Incubation in Na^+-fee medium causes reversal of the Na^+/Ca^{2+} exchange activity [6] leading to a rapid rise in $[Ca^{2+}]_i$. Using this procedure, the effect of simvastatin-sodium or simvastatin on the Na^+/Ca^{2+} exchanger was investigated.

2.7. Statistics

All data are presented as mean values ± S.E.M. Student's *t*-test was used for statistical comparison, and corrected for multiple comparison (Bonferroni method) when necessary. A *P* value less than 0.05 was considered to indicate a significant difference.

3. Results and discussion

The time–courses of LDH release from cultured cardiomyocytes incubated with or without simvastatin-sodium (20 and 50 μM) under anoxic conditions are illustrated in Fig. 1. Simvastatin-sodium delayed LDL release, which is more pronounced with 50 μM than with 20 μM simvastatin-sodium. Table 1 shows that 2 μM simvastatin-sodium did not alter the time to 50% LDH release, as compared to controls, whereas 100 μM simvastatin-sodium did not prolong the time to 50% LDH release any further compared to 50 μM simvastatin-sodium.

Table 1
Prolongation (positive values) or shortening (negative values) of the time to 50% LDH release from cardiomyocytes during anoxia by the presence of simvastatin-sodium, as compared to control cells ($\Delta LDH_{50\%}$)

Concentration simvastatin-sodium (μM)	$\Delta LDH_{50\%}$ (min)
2	−4 ± 3.9
20	30 ± 12*
50	53 ± 6.1*
100	44 ± 8.5*

Mean ± S.E.M., $n = 5$; *$P < 0.05$ compared to controls (no simvastatin-sodium).

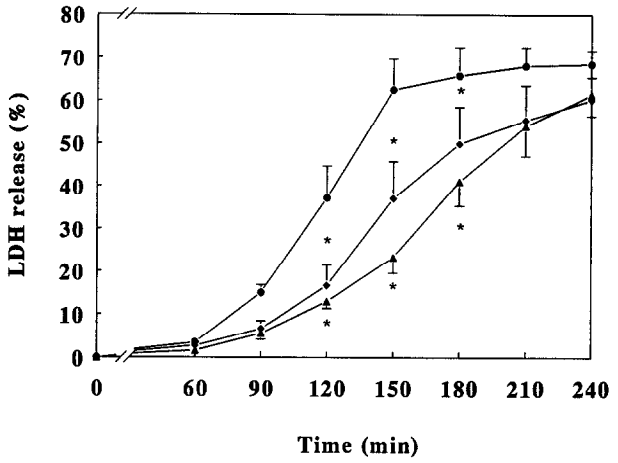

Fig. 1. Time–courses of lactate dehydrogenase (LDH) release from cultured neonatal cardiomyocytes incubated under anoxic conditions. The activity of LDH release is expressed as a percentage of the LDH activities present in the cells at $t = 0$. ●, without simvastatin-sodium; ♦, with 20 μM simvastatin-sodium; and ■, with 50 μM simvastatin-sodium. Each point represents the mean ± S.E.M. ($n = 5$). *$P < 0.05$ compared to anoxia without simvastatin-sodium.

Simvastatin-sodium could exert its protective effect by interfering with a variety of factors and pathways involved in the mechanism of cell death during anoxia [12]. Calcium overload is considered to play an important role in the development of irreversible cell damage during anoxia [6,7]. Calcium overload can lead to cell death through several pathways, including activation of calcium-dependent phospholipases, proteases and endonucleases [13]. Therefore, we have monitored the $[Ca^{2+}]_i$ in cultured myocytes during normoxia and anoxia. Under normoxic conditions 20 and 50 μM simvastatin-sodium increased the F_{340}/F_{380} ratio of Fura-2 (Fig. 2), which

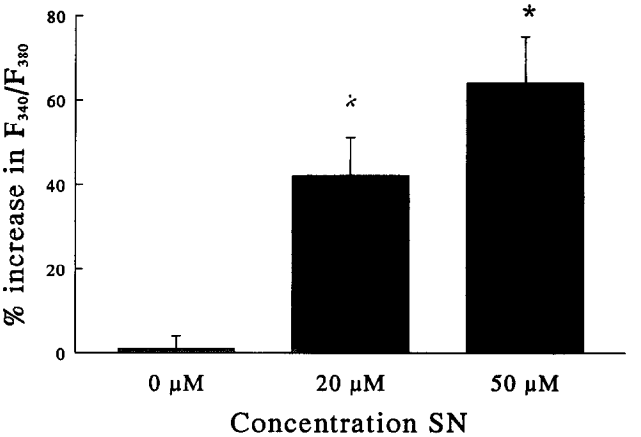

Fig. 2. Increase in F_{340}/F_{380} values of Fura-2 measured in normoxic cardiomyocytes in the presence of 20 or 50 μM simvastatin-sodium (SN). Ratio values are expressed relative to the ratio value measured before addition of 0, 20 or 50 μM simvastatin-sodium. Indicated are mean values ± S.E.M. ($n = 4$). *$P < 0.05$ compared to controls (no simvastatin-sodium).

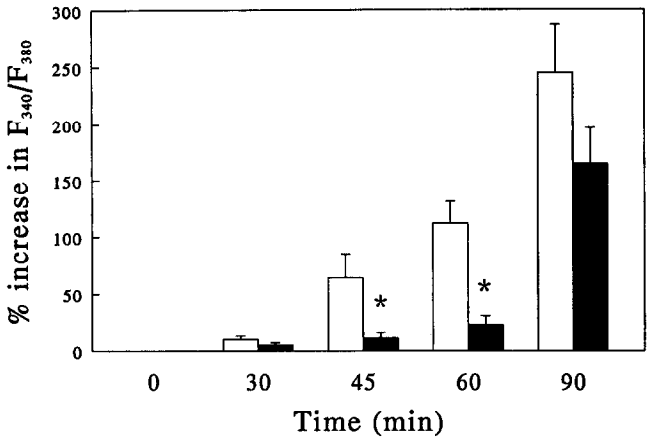

Fig. 3. Increase in F_{340}/F_{380} values of Fura-2 measured in anoxic cardiomyocytes in the absence (open bars) and presence of 50 μM simvastin-sodium (solid bars). Ratio values are expressed relative to the ratio value measured at $t = 0$, that is, at the start of anoxia. Indicated are mean values ± S.E.M. ($n = 4$). *$P < 0.05$ compared to anoxia without simvastatin-sodium.

indicates that simvastatin-sodium causes an increase in $[Ca^{2+}]_i$.

In another series of experiments, we assessed the effect of 50 μM simvastatin-sodium on $[Ca^{2+}]_i$ during anoxia. As shown in Fig. 3, the F_{340}/F_{380} ratio of Fura-2 was increased by 60% after 45 min of anoxia without simvastatin-sodium, whereas in the presence of 50 μM simvastatin-sodium no significant increase in the F_{340}/F_{380} ratio was seen after 45 min of anoxia. After 90 min of anoxia, the F_{340}/F_{380} ratio had increased by 248% without simvastatin-sodium, and by 162% in cultures incubated with 50 μM simvastatin-sodium. Unfortunately, we could not measure reliable fluorescence values of Fura-loaded cells at time points beyond 90 min of anoxia due to detachment of the myocytes from the coverslips. However, it is clear from Fig. 3 that simvastatin-sodium attenuated the increase in $[Ca^{2+}]_i$ during anoxia.

Recently, Ziegelstein et al. [6] showed that the antioxidant, dimethylthiourea (DMTU), increased $[Ca^{2+}]_i$ in normoxic myocytes but attenuated the rise in $[Ca^{2+}]_i$ during hypoxia/reoxygenation. In this respect DMTU has effects quite similar to those of simvastatin-sodium presented in this study. Ziegelstein et al. [6] showed that DMTU inhibits the Na^+/Ca^{2+} exchanger in the sarcolemma of myocytes. Under normal conditions the Na^+/Ca^{2+} exchanger pumps one Ca^{2+} ion out of the cell in exchange for three Na^+ ions, driven by the transmembrane Na^+ gradient and by the membrane potential [14]. During anoxia, membrane depolarization and an increase in intracellular Na^+ concentration favors a reversal of the direction of the Na^+/Ca^{2+} exchanger [7]. Thus, during normoxia, an inhibitor of the Na^+/Ca^{2+} exchanger will increase $[Ca^{2+}]_i$, whereas during anoxia, inhibition of the Na^+/Ca^{2+} exchange will attenuate the rise in $[Ca^{2+}]_i$. To determine whether a compound is capable of inhibiting the Na^+/Ca^{2+} exchanger, Ca^{2+} entry through the exchanger can be challenged by Na^+-free incubation while the determination of the F_{340}/F_{380} of Fura-2 reflects $[Ca^{2+}]_i$. Upon Na^+-free incubation of the cardiomyocytes, the F_{340}/F_{380} ratio rose immediately, reached its maximum value within 2 min, and remained elevated for at least 15 min. The effect of simvastatin-sodium on the rise of F_{340}/F_{380} induced by Na^+-free incubation is shown in Fig. 4. 20 and 50 μM simvastatin-sodium attenuated the rise in the F_{340}/F_{380} ratio to 30 ± 5% and 25 ± 8% of the values in untreated cells, respectively. These findings indicate that simvastatin-sodium inhibits the Na^+/Ca^{2+} exchanger, which could explain the attenuation by simvastatin-sodium of the anoxia-induced rise in $[Ca^{2+}]_i$, thereby delaying cell death.

Due to the short incubation times used in our experiments (< 1 h), the effects of simvastatin-sodium can only be explained by its acute direct action, as changes in cellular cholesterol content by HMG-CoA reductase inhibition take much longer to develop. It is important to note that the effects of simvastatin-sodium described in the present study are observed at concentrations much higher than that reached in plasma of hypercholesterolemic patients who take the anti-hyperlipidemic drug, simvastatin [15].

In conclusion, we have shown that simvastatin-sodium improves the tolerance of cultured cardiomyocytes to anoxia. The protective effect of simvastatin-sodium is mediated by its inhibitory effect on the Na^+/Ca^{2+} exchanger, thereby attenuating the rise in $[Ca^{2+}]_i$ during anoxia.

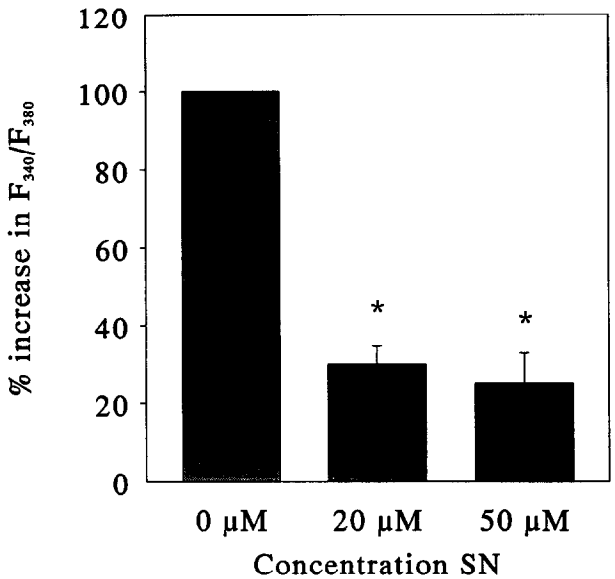

Fig. 4. Increase in F_{340}/F_{380} values of Fura-2 measured in cardiomyocytes after 10 min of Na^+-free incubation. The change in the F_{340}/F_{380} ratio observed in the absence of simvastatin-sodium (SN) is set at 100% (control). The presence of 20 and 50 μM simvastatin-sodium reduced the rise of the F_{340}/F_{380} ratio caused by Na^+-free incubation by about 70%. Means ± S.E.M., $n = 5$; *$P < 0.05$ compared to controls (no simvastatin-sodium).

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