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Vascular smooth muscle cell apoptosis induced by 7-ketocholesterol was mediated via Ca^{2+} and inhibited by the calcium channel blocker nifedipine

Hidehisa Sasaki[a], Fusako Watanabe[b], Takeyoshi Murano[b], Yoh Miyashita[c,*], Kohji Shirai[c]

[a]*Department of Pharmacy, Sakura Hospital, School of Medicine, Toho University, Chiba 285-8741, Japan*
[b]*Department of Clinical Laboratry Medicine, Sakura Hospital, School of Medicne, Tohu University, Chiba 285-8741, Japan*
[c]*Department of Internal Medicine, Sakura Hospital, School of Medicine, Toho University, Chiba 285-8741, Japan*

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Abstract

Previous reports indicate that 7-ketocholesterol (7KCHO) induces apoptosis of cultured human vascular smooth muscle cells (SMCs). We hypothesized that calcium channel blockers will inhibit SMC apoptosis induced by 7KCHO because caspase-3 activity is Ca^{2+} dependent and 7KCHO stimulates caspase-3 and SMC apoptosis. So, the protective effect of the calcium channel blocker nifedipine on SMC apoptosis induced by 7KCHO was investigated. When 7KCHO (50 μmol/L) was added to SMCs, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling was positive. DNA extracted from SMCs exposed to 7KCHO showed a ladder pattern on agarose electrophoresis. In the presence of extracellular Ca^{2+}, the Ca^{2+} influx, caspase-3 activity, and fragmented DNA also increased in SMCs incubated with 7KCHO dose-dependently. However, in the absence of extracellular Ca^{2+}, no effects of 7KCHO on caspase-3 activity and fragmented DNA were observed. In the presence of nifedipine, the 7KCHO-induced increases in Ca^{2+} influx, caspase-3 activity, and the amount of fragmented DNA decreased significantly. These results suggest that 7KCHO-induced apoptosis of SMCs is inhibited by calcium channel blockade, and that Ca^{2+} influx into cells mediated by 7KCHO plays an important role in 7KCHO-induced apoptosis.

1. Introduction

Calcium channel blockers (CCBs) are known to prevent acute coronary syndrome, and their hypotensive effects have been regarded to be an important mechanism for the preventive effect [1,2]. On the other hand, CCBs have been reported to suppress atherogenesis in nonhypertensive patients [3,4]. These reports suggest that CCBs may act directly on atherosclerotic lesions and suppress atherogenesis. As an example of direct effects of CCBs on cells, verapamil has been shown to inhibit elastase release and superoxide anion production in human neutrophils [5], but there are few in vitro studies in these fields, and the precise mechanisms of the anti-atherosclerotic effect of CCBs remain unclear.

Oxysterols are known to be present in high concentrations in atherosclerotic plaques [6], and contribute to the development of atherosclerosis [7] by various mechanisms such as cytotoxic or apoptotic potential for endothelial cells [8-10], impairment of vascular endothelial barrier function [11,12], inhibition of endothelial nitric oxide release [13], and arterial relaxation [14]. We have reported that 7-ketocholesterol (7KCHO), an oxysterol, induces apoptosis of human vascular smooth muscle cells (SMCs) [15,16] and inhibits migration of SMCs [17]. These findings suggest that accumulation of 7KCHO in atherosclerotic plaque may decrease the SMC numbers in the plaque and render atherosclerotic plaques unstable.

Activation of caspases in cells plays an important role in apoptosis [18,19]. Caspases are known to be key proteolytic mediators in the initiation and execution of the apoptotic response [20-23]. These enzymes require Ca^{2+} in their activation [24]. Therefore, if 7KCHO has the potential of inducing apoptosis of SMCs, then addition of 7KCHO to SMC cultures should result in Ca^{2+} influx and caspase-3 activation in the cells. However, few reports have examined the relation between Ca^{2+} influx and caspase-3 activity in SMCs.

We hypothesized that CCBs will inhibit SMC apoptosis induced by 7KCHO because caspase-3 activity is Ca^{2+} dependent and 7KCHO stimulates caspase-3 and SMC

* Corresponding author. Tel.: +81 43 462 8811; fax: +81 43 489 9770.
E-mail address: mumon@sf6.so-net.ne.jp (Y. Miyashita).

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apoptosis. So, in this study, we examined whether 7KCHO promotes Ca^{2+} influx and caspase-3 activity in human SMCs, and furthermore, whether CCB suppresses apoptosis induced by 7KCHO.

2. Materials and methods

2.1. Materials

7-Ketocholesterol (5-cholesten-3β-ol-7-one) and cholesterol were purchased from Sigma Chemical (Steinheim, Germany). The calcium channel blocker nifedipine was provided by Bayer (Jerusalem, Israel). Fetal bovine serum (FBS) and Dulbecco modified Eagle medium (DMEM) were obtained from Gibco (Tokyo, Japan).

2.2. Cell culture

Smooth muscle cells were obtained from human coronary arteries at postmortem examinations. Briefly, the vascular wall containing the medial and intimal layers was obtained from human coronary arteries, and the endothelial and adventitia layers were stripped by a razor carefully. Pieces (1-2 mm^3) of the media were placed in T-25 flasks. Complete DMEM with 10% FBS and 1% gentamicin was added. The medium was changed 2 or 3 times per week. After approximately 4 weeks, cells were harvested from the flasks with trypsin-EDTA (Gibco) followed by centrifugation (200g for 5 minutes). The cell pellet was resuspended in DMEM with 10% FBS and plated into T-75 flasks. In all experiments, cells were cultured in DMEM (100 mg/dL of glucose) containing 100 U/mL penicillin and 100 μg/mL streptomycin, and equilibrated in 95% air/5% CO_2. The purity and identity of SMCs were verified using a monoclonal antibody against smooth muscle α-actin. Cells at passage 3 were used in all experiments.

2.3. Detection of DNA nicks

Cells (1.2×10^4 per well) were plated onto chamber slides (Nalga Nunc International, NY, USA) and cultured in DMEM with 10% FBS for 72 hours. Then, cholesterol or 7KCHO was added at 50 μmol/L. After incubation for 24 hours, DNA nicks that are characteristic in apoptosis were detected by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end-labeling (TUNEL) [25] using an Apotag in situ apoptosis detection kit (s7100-kit from Oncor, Gaithersburg, MD.).

2.4. Agarose electrophoresis

Cells (1.2×10^5 per well) were plated into 12-well plates and cultured in DMEM with 10% FBS for 72 hours. Then, cholesterol or 7KCHO was added at 50 μmol/L. After incubation for 24 hours, DNA fragmentation was detected by agarose electrophoresis. Suspended cells and adherent cells were harvested by centrifugation at 200g for 10 minutes. Cell pellets were washed with DMEM and disrupted in a high-molecular-weight buffer (pH 7.5) (150 μmol/L NaCl, 10 μmol/L Tris-HCl, 10 μmol/L EDTA) containing 0.1% sodium dodecyl sulfate and 0.1% proteinase K. After incubation for 90 minutes at 55°C, samples were extracted twice with phenol/chloroform (1:1, vol/vol) and digested with ribonuclease A (1 mg/mL) at 37°C for 60 minutes. DNA was then precipitated with ethanol. The samples were kept at −70°C for 20 minutes. They were then centrifuged at 15 000g for 20 minutes, rinsed with 70% ethanol, and dried under vacuum. The DNA was dissolved in Tris-EDTA buffer, pH 7.4 (10 μmol/L Tris, 1 μmol/L EDTA) at 4°C for 24 hours. Loading buffer containing 50% glycerol, 5 × TAE (1 × TAE = 40 mmol/L Tris acetate, pH 8.5, 2 mmol/L EDTA), and 0.1% bromophenol blue was added to the samples at a ratio of 1:4 (vol/vol). Samples were run on a 1.8% agarose gel at 10 V/cm in TAE

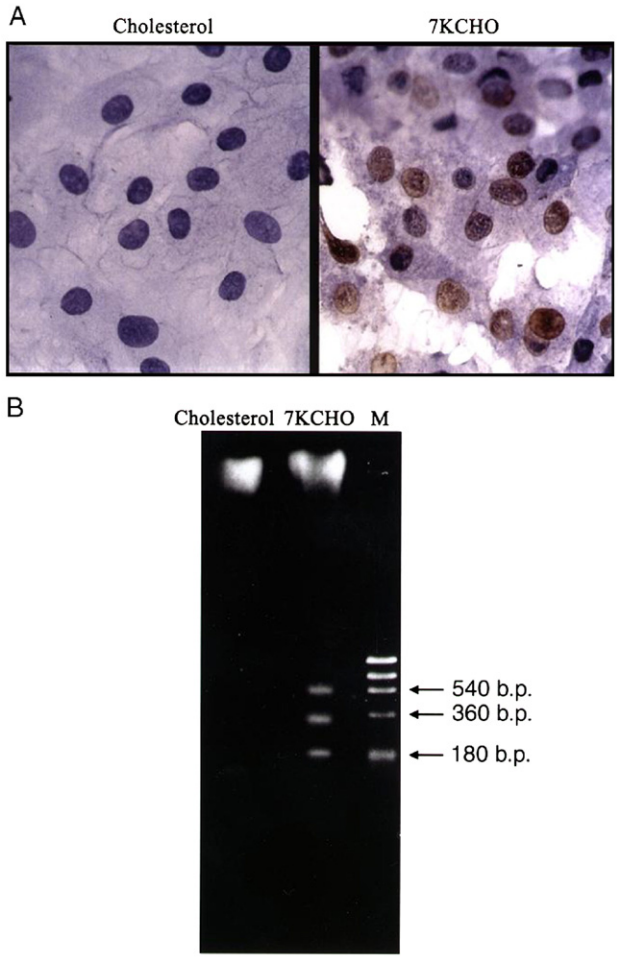

Fig. 1. TUNEL staining (A) and agarose electrophoresis of DNA (B) of vascular smooth muscle cells after incubation with cholesterol or 7KCHO. Cells at passage 3 were seeded onto a chamber slide and cultured in DMEM with 10% FBS for 72 hours. Then, cholesterol or 7KCHO at 50 μmol/L was added and TUNEL staining was performed after incubation for 24 hours (original magnification ×600). For agarose electrophoresis, 1.2×10^5 cells per well were plated into 12-well plates. After culturing in DMEM with 10% FBS for 72 hours, cholesterol or 7KCHO was added at 50 μmol/L. After incubation for 24 hours, agarose electrophoresis was performed. M indicates marker of molecular weight.

buffer. DNA was visualized on an ultraviolet transilluminator after staining with ethidium bromide (0.5 mg/mL).

2.5. Measurement of intracellular Ca^{2+}

The Ca^{2+} responses in SMCs were assessed using fluo-3-AM in conjunction with a fluorometric imaging plate reader (Molecular Devices, Chiba, Japan). Cells (1.2×10^4 per well) were plated into 12-well plates and grown in DMEM with 10% FBS for 72 hours. Fluo-3 loading was achieved by exposing the cells to 5 μmol/L fluo-3-AM in Hanks-based HEPES buffer (Calcium Screening Kit II, Dojindo, Kumamoto, Japan) containing 0.05% (wt/vol) Pluronic F-127. The plate was immediately placed on the fluorometric imaging plate reader and 50 μmol/L of 7KCHO was added at 1 minute. The relative fluorescence change of fluo-3 was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm. Nifedipine was added at 0.1 or 1.0 μmol/L 24 hours before adding 7KCHO.

2.6. Measurement of caspase-3 activity

Cells (1.2×10^5 per well) were plated into 12-well plates and grown in DMEM with 10% FBS for 72 hours. Then, the medium was changed to Ca^{2+}-free or Ca^{2+}-containing phosphate-buffered saline (PBS), and 7KCHO was added at varying concentrations. After incubation for 24 hours, the activity of caspase-3 was measured. Nifedipine was added at 0.1 or 1.0 μmol/L 24 hours before adding 7KCHO. Activity of caspase-3 was measured using a commercially available kit (CaspACE Assay System, Promega, Madison, WI) following the recommendations of the manufacturer. After cells were centrifuged at 200*g* for 10 minutes, cell pellets were kept on ice. Cell pellets were washed with ice-cold PBS and suspended in cell lysis buffer. Cell lysates were incubated on ice for 15 minutes. After centrifugation at 15 000*g* for 20 minutes, the supernatant was collected. An equal volume of assay reagent DEVD-pNA and a fluorometric substrate of caspase-3 were added to the cell culture and incubated at 37°C for 4 hours. The fluorescence of cleaved pNA was measured by spectrofluorometry on an automatic microtiter plate reader (Microplate Reader EZS-ABS, Asahi Techno Glass, Tokyo, Japan) at an excitation wavelength of 405 nm. Data were corrected using the quantity of total protein in the well.

2.7. DNA fragmentation assay

Amount of fragmented DNA was assayed as described previously [26]. Cells (1.2×10^4 per well) were plated into 12-well plates and cultured in DMEM with 10% FBS for 24 hours. Then, the DNA of cells was labeled with 5-bromo-2′-deoxy-uridine (BrdU) for 48 hours. Thereafter, the medium was changed to Ca^{2+}-free or Ca^{2+}-containing PBS, and 7KCHO was added at varying concentrations. After incubation for 24 hours, the amount of BrdU-labeled DNA released into the supernatant by dead cells was measured by a quantitative sandwich enzyme immunoassay (Cellular DNA Fragmentation ELISA, Boehringer Mannheim, Mannheim, Germany, cat. no. 1585045). Nifedipine was added at 0.1 or 1.0 μmol/L 24 hours before adding 7KCHO. Data were corrected using the quantity of total protein in the well, which was measured by bicinchoninic acid protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL).

2.8. Statistical analysis

Paired analysis between 2 groups was performed using Student *t* test, when analysis of variance indicated significance for multiple comparison. Data are expressed as mean ± SD. *P* values of less than .05 were considered significant.

3. Results

3.1. TUNEL staining and DNA laddering in SMCs incubated with cholesterol and 7KCHO

DNA nicks were detected by the TUNEL method. Cells incubated with 7KCHO stained positive, but cells incubated with cholesterol showed negative staining by TUNEL

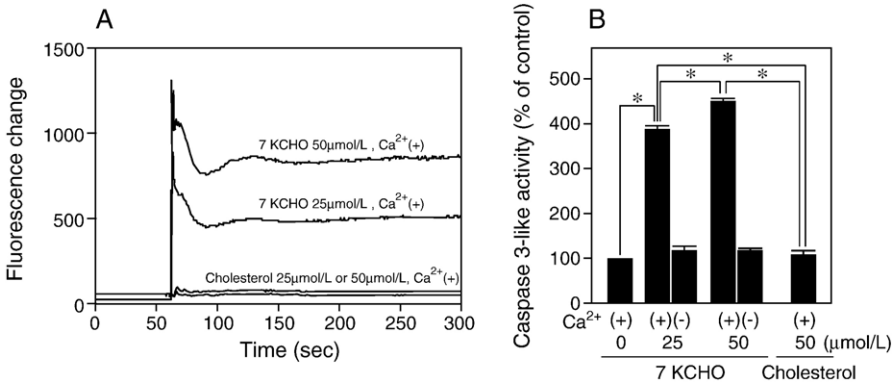

Fig. 2. Effects of 7KCHO on Ca^{2+} influx (A) and caspase-3 activity (B) in vascular smooth muscle cells. A, Cells (1.2×10^5 per well) were plated into 12-well plates and grown in DMEM with 10% FBS for 72 hours. Then, the medium was changed to Ca^{2+} containing PBS, and 7KCHO or cholesterol was added at 25 or 50 μmol/L. After that, intracellular Ca^{2+} concentration was measured immediately. B, Cells (1.2×10^5 per well) were plated into 12-well plates and grown in DMEM with 10% FBS for 72 hours. Then, the medium was changed to Ca^{2+}-free or Ca^{2+}-containing PBS, and 7KCHO or cholesterol was added at 25 or 50 μmol/L. After incubation for 24 hours, the activity of caspase-3 was measured. Results are shown as percentage of control, which is caspase-3–like activity recognized without 7KCHO. Values represent mean ± SEM of 2 experiments performed in triplicate. *$P < .001$.

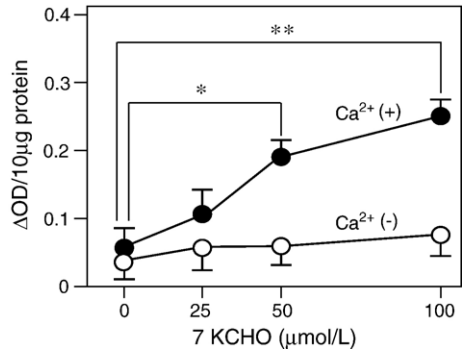

Fig. 3. The effect of various concentrations of 7KCHO on the amount of DNA fragmentation in SMCs. Cells (1.2×10^5 per well) were plated into 12-well plates. After growing in DMEM with 10% FBS for 24 hours, BrdU was added and incubated for 48 hours. Then, the medium was changed to Ca^{2+}-free PBS (open circle) or Ca^{2+}-containing PBS (closed circle), and 7KCHO was added at varying concentrations. After incubation for 24 hours, DNA fragmentation assay was performed. Results are the mean ± SEM of 2 experiments performed in triplicate. *$P < .05$; **$P < .01$.

(Fig. 1A). DNA from SMCs incubated with cholesterol or 7KCHO at 50 μmol/L was extracted and subjected to agarose electrophoresis. The DNA from SMCs incubated with 7KCHO showed a ladder pattern (monomer size of 180 base pairs) (Fig. 1B). Cells incubated with cholesterol showed no ladder pattern (Fig. 1B). The results of TUNEL staining and DNA laddering indicated that 7KCHO induced apoptosis of SMCs.

3.2. Effects of 7KCHO on Ca^{2+} influx and the activity of caspase-3

In the presence of extracellular Ca^{2+}, incubation of SMCs with 7KCHO caused Ca^{2+} influx in SMCs (Fig. 2A), and the amount of Ca^{2+} influx induced by 50 μmol/L of 7KCHO was higher than that by 25 μmol/L of 7KCHO (Fig. 3A). However, no Ca^{2+} influx was observed by the addition of 25 or 50 μmol/L of cholesterol. In the presence of extracellular Ca^{2+}, the activity of caspase-3 in SMCs incubated with 25 μmol/L of 7KCHO showed a significant increase by about 400% compared with SMCs incubated with cholesterol, and a dose-dependent increase was observed with 7KCHO (Fig. 2B). By the addition of 50 μmol/L of cholesterol, no activity of caspase-3 was observed (Fig. 2B). In the absence of extracellular Ca^{2+}, no caspase-3 activity was observed even if 7KCHO was added (Fig. 3B).

3.3. Quantitative analysis of apoptosis induced by 7KCHO

Fragmented DNA assay was performed by enzyme-linked immunosorbent assay [15,16,26] to analyze apoptosis quantitatively. In the presence of extracellular Ca^{2+}, an increase in amount of fragmented DNA was observed with the addition of 7KCHO in a dose-dependent manner, but no increase in fragmented DNA was recognized with Ca^{2+}-free condition (Fig. 3). The increases recognized in the presence of extracellular Ca^{2+} were significant compared with that recognized in Ca^{2+}-free condition at 7KCHO concentrations of 50 and 100 μmol/L.

3.4. The effect of nifedipine on Ca^{2+} influx and the activity of caspase-3 induced by 7KCHO

Next, to clarify the effect of CCB on apoptosis induced by 7KCHO, we observed Ca^{2+} influx, caspase-3 activity, and the amount of fragmented DNA induced by 7KCHO

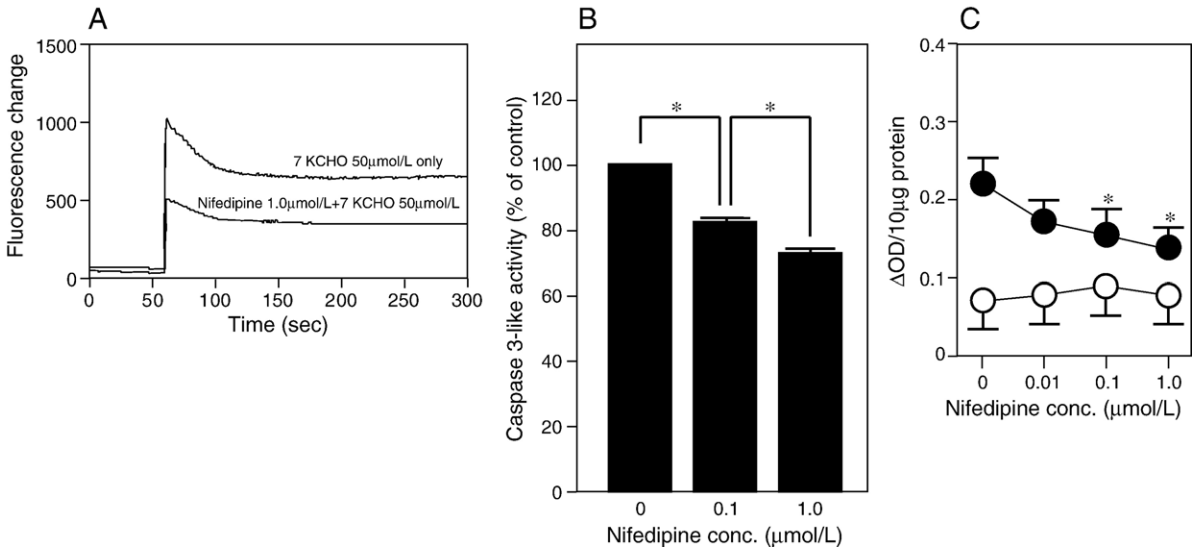

Fig. 4. The inhibitory effect of nifedipine on Ca^{2+} influx (A), caspase-3 activity (B), and fragmented DNA (C) induced by 7KCHO in vascular smooth muscle cells. Cells (1.2×10^5 per well) were plated into 12-well plates and grown in DMEM with 10% FBS for 48 hours. Then, nifedipine was added at 0.1 or 1.0 μmol/L. After the addition of nifedipine, the cells were incubated for 24 hours, and then the medium was changed to Ca^{2+}- and nifedipine-containing PBS, and 7KCHO was added at 50 μmol/L. A, After that, intracellular Ca^{2+} concentration was measured immediately. B and C, After incubation for another 24 hours, caspase-3 activity measurement and DNA fragmentation assay were performed. Closed circles indicate incubation in the presence of 7KCHO; open circles, incubation in the absence of 7KCHO. Results of caspase-3–like activity are shown as percentage of control, which is a value recognized without nifedipine. Results of all experiments are the mean ± SEM of 2 experiments performed in triplicate. *$P < .001$.

when nifedipine was added. Ca^{2+} influx induced by 7KCHO was suppressed in the presence of nifedipine (Fig. 4A). By the addition of 0.1 μmol/L of nifedipine, the activity of caspase-3 induced by 7KCHO showed a significant decrease by about 30%, and a dose-dependent decrease was observed with nifedipine (Fig. 4B). The amount of fragmented DNA induced by 7KCHO significantly decreased with the addition of nifedipine in a dose-dependent manner (Fig. 4C). With the addition of nifedipine alone to SMCs, there were no effects on the activity of caspase-3 and fragmented DNA (data not shown). These findings indicated that nifedipine suppressed SMC apoptosis induced by 7KCHO.

4. Discussion

In atherosclerotic lesions, apoptosis of SMC is observed and related to plaque rupture [9,10]. In the present study, the results of TUNEL and DNA laddering indicate that 7KCHO induces apoptosis of SMC. 7-Ketocholesterol is believed to participate in the initiation and/or progression of atherosclerosis because 7KCHO accumulates in atherosclerotic lesions [6] and is capable of inducing SMC dysfunction including apoptosis [15,17]. We already reported that apoptosis of SMCs induced by 7KCHO is accelerated when SMCs acquire a high proliferative potential [26]. If 7KCHO accelerates SMC apoptosis in atherosclerotic plaque, this results in a reduction of cell number and may consequently render the plaque fragile, leading to plaque rupture. Therefore, clarification of the mechanism on how 7KCHO induces apoptosis may provide a clue to decrease the incidence of acute coronary disease.

Ca^{2+} influx into cells plays an important role in the process of apoptosis caused by several compounds in many types of cells [24]. Caspases are known to be key proteolytic mediators in the initiation and execution of the apoptotic response [20-23], and these enzymes require Ca^{2+} for activation. The present study demonstrated that 7KCHO increased intracellular Ca^{2+} in human SMC, and consequently caspase-3 activity was increased. However, in the absence of extracellular Ca^{2+}, no caspase-3 activity was observed even if 7KCHO was added. These results indicate that 7KCHO has the potential to induce $Ca2^{+}$ influx into cells, and this potential could be one of the important mechanisms of 7KCHO-induced apoptosis.

The present study also showed that nifedipine decreased Ca^{2+} influx into SMCs by 7KCHO. This result indicates that 7KCHO-induced Ca^{2+} influx is mediated through the Ca channel because nifedipine is a calcium channel blocker. The activity of caspase-3 and the amount of DNA fragmentation induced by 7KCHO were decreased by nifedipine. These results suggest that the suppression of Ca^{2+} influx into cells reduces apoptosis induced by 7KCHO.

Upon incubation with 0.01 μmol/L of nifedipine, the amount of fragmented DNA induced by 7KCHO decreased significantly. When clinical doses of nifedipine are taken daily, the Cmax of nifedipine is approximately 0.1 μmol/L [27,28]. Accordingly, the apoptosis-inhibiting effect of CCB observed in vitro could occur in the body of patients who take CCBs everyday for the treatment of hypertension.

Several studies have shown that CCBs have an antiatherosclerotic action not only in animals but also in humans [3,29]. This action may be a result of pleiotropic effects beyond the blood pressure–lowering effect, although controversies remain. The pleiotropic effects of CCBs are supported by studies showing that CCBs inhibit smooth muscle cell proliferation [30] and decrease very low-density lipoprotein secretion by hepatocytes [3,4]. In the present in vitro study, CCBs at clinically achievable concentrations suppressed SMC apoptosis induced by 7KCHO. This effect may stabilize atherosclerotic plaques by inhibiting the decrease in cell number in atherosclerotic lesion and may be one of the pleiotropic effects contributing to the antiatherosclerotic action recognized in CCBs.

In summary, our study suggests that CCB inhibits 7KCHO-induced apoptosis of SMCs, and one of mechanisms may be through inhibiting the activation of caspases in SMCs by decreasing Ca^{2+} influx into SMCs.

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