Effects of Leonurine on L-Type Calcium Channel in Rat Ventricular Myocytes

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Leonurine (Leo) is a special alkaloid principle of Herba leonuri that has recently been suggested to improve cardiovascular functions. To date, there is no direct ionic evidence of Leo on regulating calcium channels in the heart. In the present study, we examined the effects of Leo on action potentials and membrane currents recorded from isolated rat ventricular myocytes with the whole-cell patch clamp technique. Leo 100 μM shortened the action potential duration in a dose-dependent manner. Leo up to 200 μM had no significant effect on the Na+ current (INa) and K+ current (IK). However, Leo depressed the L-type Ca2+ current (ICa,L). In the presence of 20 and 100 μM Leo, the current density was decreased and the voltage at half maximal inactivation V0.5 shift to more negative potential. The recovery time constant was also delayed. In addition, the transcription and protein expression levels of L-type calcium channel (CaV1.3) in primary cultured neonatal myocytes from Sprague-Dawley rats were reduced by Leo treatment in a dose-dependent fashion as assessed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assays. We conclude that Leo inhibits L-type calcium channels in cardiomyocytes.

Key words Herba leonuri; action potential; action potential duration; calcium channel; cardiac ventricle

Herba leonuri (HL, also called as Chinese Motherwort) is a well-known herbal medicine in China. Traditionally, the beneficial effects of the herb on uterus system have been extensively investigated. Recent studies have indicated that this herb has versatile effects, especially in the field of cardiovascular diseases. Studies have shown that HL extracts ameliorated myocardial ischemia and improved heart functions. Leonurine (Leo, 4-guanidino-n-butyl syringate, also named as SCM-198) is the special alkaloid for HL. Our group carried out a series of studies focusing on its cardioprotective effect. Our findings indicated that Leo could attenuate both doxorubicin and hypoxia induced apoptosis in H9c2 cardiac muscle cells, decrease infarct size in acute myocardium ischemia and improve cardiac recovery during chronic infarction. Using middle cerebral artery occlusion model we also found Leo had neuroprotective effects. The antioxidative and anti-apoptotic effects were, at least in part, involved in the underlying mechanism. But other mechanisms remain elusive.

Calcium (Ca2+) is an important signal molecule for regulating cardiac myocytes function. The fundamental role is to enable excitation-contraction coupling. While a small amount of Ca2+ is necessary for physiological function, an increased cytosolic free Ca2+ overload is demonstrated to be a major mechanism of myocardial ischemia/reperfusion (I/R) induced injury. Calcium handling machinery has become a prime target to improve post-ischemic myocardial function at injury. Voltage-gated calcium channels (VGCCs) play a key role in transducing cell surface membrane potential with local intracellular Ca2+ pathways. Among VGCCs, L-type Ca2+ channels (LTCC) represent a well-established therapeutic target for calcium channel blockers, which are widely used to treat hypertension and myocardial ischemia. Four isoforms have been identified and classified from CaV1.1 to CaV1.4. Heart tissue expresses CaV1.2 and CaV1.3. The major component of the LTCC is the pore-forming α1 subunit, which contains the binding site for calcium channel blockers, the voltage-sensor, and the selectivity filter. Subunit α1C is the primary isoform found in the heart and gives rise to high-voltage-activated L-type Ca2+ currents (I Ca,L). Ca2+ overload in cardiomyocytes could induce mitochondrial Ca2+ overload and depletion of tissue ATP stores. Our previous studies have shown that Leo decreased intracellular Ca2+ overload and exerted mitochondrial protective effect both in cardiomyocytes and brain. But little was known about the mechanism underlying this phenomenon.

In the present study, we examined the effect of Leo on cardiac action potential (AP), and found that it decreases the AP duration in isolated rat ventricular myocytes. We further performed detailed electrophysiological and biological investigations to ascertain the target channel of Leo. We characterized the effects of Leo on the L-type Ca2+ currents (I Ca,L) in ventricular myocytes of neonatal and adult rats. Our study firstly investigated the effects of Leo on LTCC, and consequently may contribute to its cardioprotective effects.

MATERIALS AND METHODS

Chemical Synthesis of Leo Leo was synthesized from syringic acid by carbonylation, reaction with SOCl2, Gabriel reaction et al. The structure of molecular was identified by 1H-NMR, 13C-NMR and electron ionization (EI)-MS. HPLC analysis showed the purity was >99%.

Animals Neonatal and adult Sprague-Dawley rats were obtained from the Department of Experimental Animals, Fudan University. The investigation conformed to the Guide
for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) in the U.S.A. and was approved by the Ethics Committee of Experimental Research, Fudan University.

**Adult Rat Myocytes Preparation** Freshly isolated single ventricular myocytes were prepared from the hearts of adult male Sprague-Dawley rats (200 to 250 g) as previously described. Briefly, hearts were rapidly excised and retrogradely perfused on a Langendorff perfusion apparatus, at 37°C, via the aorta with an oxygenated buffer solution followed by a Ca²⁺-free Tyrode solution with the following composition (mm): NaCl 135, KCl 5.4, NaH₂PO₄ 0.33, MgCl₂ 1, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) 10, CaCl₂ 0.2, and glucose 10 (pH 7.4 in 95%O₂–5%CO₂). Hearts were then perfused with a collagenase-containing solution (1 mg/mL) and rinsed with Ca²⁺-free Tyrode solution. Isolated ventricular myocytes were mechanically dispersed with a wide-bore pipette in modified Kraf–Bruehe (KB) solution at room temperature. The modified KB solution containing (mm): l-glutamic acid 50, KOH 80, KCl 40, MgSO₄ 3, KH₂PO₄ 25, HEPES 10, ethylene glycol bis(2-aminoethyl)ether-N,N,N',N'-tetraacetic acid (EGTA) 1, taourine 20 and glucose 10, pH 7.4. The cell suspension was stored at 4°C in modified KB solution. Rod-shaped myocytes were used between 1 and 8 h after isolation.

**Electrophysiological Recordings** Patch pipettes (2–5 MΩ when filled with internal solutions) were pulled from borosilicate glass capillaries. We used conventional whole cell patch clamp technique to record membrane current or voltage from single ventricular myocytes. In current-clamp mode, APs were evoked by a brief suprathreshold current pulse. Filtered signals (5 kHz) from a patch-clamp Axopatch-200B amplifier (Axon Instruments, U.S.A.) were fed into AD/DA converter (DigiData-1200 interface, Axon Instruments, U.S.A.), digitized at 10 KHz and stored in PC for later analysis. Voltage command protocols were provided by pClamp 6.0 software package (Axon Instruments, U.S.A.). Cell capacitance was measured using a short hyperpolarizing ramp pulse (5 mV in 5 ms) from a holding potential of −40 mV. The flow rate of the perfusion solution was 1.5–2 mL/min. All electrophysiological experiments were performed at room temperature (23–25°C). Different concentration of Leo was added to the bath solution for about 5 min to start to record the currents and the recording lasted for about 20 min.

All currents were recorded using the whole-cell patch clamp technique. The external solution used to record Iₖ,₅,L contains (mm): tetraethylammonium chloride (TEA-Cl) 120, HEPES 10, MgCl₂ 1, TTX 0.001, CsCl 10, CaCl₂ 2 and glucose 10, pH 7.4 (TEAOH) and that for recording Iₙa contained the following (mm): KCl 120, MgCl₂ 1, CaCl₂ 0.018, EGTA 0.5, HEPES 10 and Na₄ATP 5, pH 7.4.

Current of Iₖ was stimulated from a holding potential of −40 mV, with a series of 4.5 s depolarizing steps from −40 mV to +50 mV (with 10 mV increments), then back to −40 mV. The amplitude of Iₖ,tail was measured with a 1 s latency following the end of the depolarizing step. Current of Ito was stimulated from a holding potential at −80 mV with a series of 300 ms depolarizing steps from −60 mV to +50 mV (with 10 mV increments), then back to −80 mV. Whole-cell Iₙa was stimulated from a holding potential of −100 mV, with 30 ms depolarizing steps to −20 mV then back to −100 mV. The I–V relationship of Iₙa was stimulated from a holding potential of −100 mV to test potentials ranging from −80 to +40 mV in 10 mV step increments.

**Neonatal Rat Cardiomyocyte (NRVM) Isolation and Culture** NRVM were isolated from 2-d-old Sprague-Dawley rats by enzymatic digestion and separated from non-muscle cells as previously described. A total of 2–4 million viable myocytes were isolated per ventricle with very little fibroblast contamination (<2%) and plated in serum-containing Dulbecco’s modified Eagle’s medium (DMEM). Myocytes were incubated for 48–72 h to allow attachment and spreading, after which the medium was replaced with serum free media for 24 h prior to treatment. Then different concentration of Leo was added to the serum free medium for 12 h to test the calcium channel protein expression.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** To determine the α1c subunit of L-type calcium channel expression level, total RNA was extracted from ventricular myocytes by using Trizol reagent (Sangon, Shanghai, China) and then analyzed by a RT-PCR method. RT-PCR (one step RNA PCR kit, Sangon, Shanghai, China) was performed with a primer specific for the cardiac L-type calcium channel subunit α₁c (forward: 5'-CGC ACA CAG AAG ACA GGGT-3', reverse: 5'-CGT GTA CTC CGGT AGGTTG-3') with the myocardial RNA as a template. The RT-PCR products were analyzed by electrophoresis on 1% agarose gels. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals were used as internal controls.

**Western Blot Analysis of L-Type Calcium Channel Protein Expression** To isolate proteins, NRVM were washed once with phosphate buffered saline (PBS), then collected in protein lysis buffer with a protease-inhibitor and centrifuged at 4°C. Protein concentrations were measured by bichinchonic acid (BCA) assay. Proteins were denatured in sodium dodecyl phosphate (SDS) loading buffer, electrophoresed on 7.5% SDS-polyacrylamide gels using a mini-Protein cell (Bio-Rad, Hercules, CA, U.S.A.) and then transferred to polyvinylidene(di)fluoride (PVDF) membrane (Millipore Corporation). For immunodetection, membranes were first incubated with primary antibody (anti-cardiac α₁c, subunit, 1:200, Alomone Labs) overnight at 4°C and with secondary horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Cell Signaling, Danvers, MA, U.S.A.) for 1 h at room temperature. Immunoreactive bands were visualized via Western blotting detection system (Alpha Innotech, U.S.A.) using the chemiluminescence method. AlphaEase (Alpha Innotech, U.S.A.) software was used to quantify band intensity. Graphs depict Caₙa,₁,₂ proteins relative to β-actin on the same blot.

**Chemicals Solutions and Drugs** Most reagents were...
purchased from Sinopharm Chemical Reagent Co. (China). ATP (Sigma), GTP (Sigma) and TEA (Sangon) were directly added when needed. To prepare a stock solution of Leo, it was dissolved in distilled water and stored at −20°C. Since the pharmacological effects of Leo decayed with time, stock solutions were used within 24 h.

Statistics Values are presented as mean±S.E.M. Data obtained from the same myocyte were used to express the results in terms of percentage. Statistical significance was evaluated by the two-tailed paired Student’s t-test. When more than one test concentration was compared, data were evaluated by one-way analysis of variance (ANOVA). Differences at a value of \( p<0.05 \) were considered significant.

RESULTS

Leo Shortened AP in Rat Ventricular Myocytes To investigate the effects of Leo on the electrical activity of the rat ventricular myocytes, we observed changes in AP shapes during the application of Leo to the bath solution. AP was recorded in current clamp mode with a whole-cell configuration. The effects of Leo on action potential duration (APD) were quantified in terms of APD\(_{50} \) and APD\(_{90} \), defined as the APD measured at the voltage where repolarization process is 50% and 90% complete. Single AP recorded in control conditions and those recorded in the presence of Leo at various concentrations were displayed in Fig. 1A. Leo markedly decreased the plateau phase. No changes in amplitude of the APs were observed during the Leo exposure, nor the resting membrane potential (RMP). The mean values for the amplitude of the APs were 125.1±3.2, 123.7±4.7, 123.2±4.5, and 121.3±5.4 mV (n=6) at control, following the application of 20 and 100 \( \mu \)M Leo, and the washout period in myocytes, respectively. The mean values for the RMP of the APs were −74.8±1.5, −74.1±2.1, −73.6±3.2, and −73.5±3.6 mV (n=6) at control, following the application of 20 and 100 \( \mu \)M Leo and the washout period in myocytes, respectively. The mean values of steady-state APD\(_{50} \) and APD\(_{90} \) obtained from six myocytes at various concentrations of Leo are summarized in Fig. 1B. Bath application of 20 and 100 \( \mu \)M Leo affected neither the maximal activation voltage nor the peak amplitude of \( I_{Na} \). A group data of six ventricular myocytes indicated that 200 \( \mu \)M Leo caused a small, but insignificant reduction in the peak amplitude of \( I_{Na} \) (23.01±2.41 pA·pF\(^{-1} \)) in control vs. 22.17±2.51 pA·pF\(^{-1} \) in Leo, n=6, \( p>0.05 \)). As shown in Fig. 2B, the superimposed I–V traces indicated that the peak amplitude of \( I_{Na} \) in control condition and that in the presence of 200 \( \mu \)M Leo recorded from the same ventricular myocyte. Leo had no significant effect on the peak amplitude of \( I_{Na} \), as well as the washout period in myocytes, respectively. The mean values for the amplitude of the APs were -125.1±3.2, -123.7±4.7, -123.2±4.5, and -121.3±5.4 mV (n=6) at control, following the application of 20 and 100 \( \mu \)M Leo, and the washout period in myocytes, respectively. The mean values for the RMP of the APs were −74.8±1.5, −74.1±2.1, −73.6±3.2, and −73.5±3.6 mV (n=6) at control, following the application of 20 and 100 \( \mu \)M Leo and the washout period in myocytes, respectively.

Leo Had No Effect on the \( I_{Na} \) Figure 2A shows representative result of \( I_{Na} \) under control condition and that in the presence of 200 \( \mu \)M Leo recorded from the same ventricular myocyte. Leo had no significant effect on the peak amplitude of \( I_{Na} \). A group data of six ventricular myocytes indicated that 200 \( \mu \)M Leo caused a small, but insignificant reduction in the peak amplitude of \( I_{Na} \) (23.01±2.41 pA·pF\(^{-1} \)) in control vs. 22.17±2.51 pA·pF\(^{-1} \) in Leo, n=6, \( p>0.05 \)). As shown in Fig. 2B, the superimposed I–V traces indicated that the peak amplitude of \( I_{Na} \) was reduced slightly by 200 \( \mu \)M Leo in the range from −50 to −10 mV, while they were similar in other ranges. Leo affected neither the maximal activation voltage nor the reversal potential. These results strongly indicate that Leo has little effect on \( I_{Na} \).

Lack of Effect of Leo on \( I_{K} \) in Ventricular Myocytes

(A) Representative AP records at various concentrations of Leo (control; 20 \( \mu \)M; 100 \( \mu \)M; wash out of Leo). AP records were obtained from the average of five sequential APs before and 1 min after bath application of 20 or 100 \( \mu \)M Leo, as well as after a 5 min washout period. (B) APD\(_{50} \) and APD\(_{90} \) (AP duration at 50% and 90% repolarization) records at various concentrations of Leo (control; 20 \( \mu \)M; 100 \( \mu \)M; wash out of Leo). Mean values of APD\(_{50} \) and APD\(_{90} \) at each concentration of Leo obtained from six different cells were showed. Asterisks indicate statistical significance (*\( p<0.05 \), **\( p<0.01 \)).

Fig. 1. Effects of Leo on Action Potential (AP) in Rat Ventricular Myocytes

(A) Representative current traces of \( I_{Na} \) in control and in the presence of 200 \( \mu \)M Leo at the same test potential for comparison in a representative myocyte. Whole-cell \( I_{Na} \) was stimulated from a holding potential of −100 mV, with 30 ms depolarizing steps to −20 mV then back to −100 mV. (B) The I–V relationships represent the peak \( I_{Na} \) values before (open square) and after (closed square) the application of 200 \( \mu \)M Leo. \( I_{Na} \) was stimulated from a holding potential of −100 mV to test potentials ranging from −80 to +40 mV in 10 mV step increments.

Fig. 2. Lack of Effect of Leo on \( I_{Na} \) Current (\( I_{Na} \)) in Rat Ventricular Myocytes

(A) Representative current traces of \( I_{Na} \) in control and in the presence of 200 \( \mu \)M Leo at the same test potential for comparison in a representative myocyte. Whole-cell \( I_{Na} \) was stimulated from a holding potential of −100 mV, with 30 ms depolarizing steps to −20 mV then back to −100 mV. (B) The I–V relationships represent the peak \( I_{Na} \) values before (open square) and after (closed square) the application of 200 \( \mu \)M Leo. \( I_{Na} \) was stimulated from a holding potential of −100 mV to test potentials ranging from −80 to +40 mV in 10 mV step increments.
Figure 3A shows that application of 200 µM Leo had no significant effect on $I_K$ in a representative myocyte. The mean $I-V$ curve of $I_K$ ($n=6$) was shifted downward slightly by 200 µM Leo (Fig. 3B). The difference, however, was not statistically significant. In control and in the presence of 200 µM Leo, the mean amplitude of $I_K$ at $+50$ mV was 6.92±0.60 pA·pF$^{-1}$ and 6.75±0.65 pA·pF$^{-1}$ ($n=6$, $p>0.05$), respectively. Leo caused small increase in the amplitude of the tail current ($I_{K,tail}$) provoked in steps to more hyperpolarized range below 30 mV, while it slightly reduced $I_{K,tail}$ in the more depolarized range (Fig. 3C). In control and in the existence of 200 µM Leo, the mean amplitude of $I_{K,tail}$ in step to $+50$ mV was 1.33±0.29 pA·pF$^{-1}$ and 1.32±0.25 pA·pF$^{-1}$ ($n=6$, $p>0.05$), respectively. Figure 3D shows that application of 200 µM Leo had no significant effect on $I_{to}$ in a representative myocyte.

**Effects of Leo on L-Type Ca$^{2+}$ Current ($I_{Ca,L}$)** Whole-cell voltage clamp experiments were conducted in adult rat myocytes. Na$^+$ currents were inactivated at the holding potential of −40 mV and blocked by TTX. K$^+$ currents were suppressed via adding Cs$^{+}$ and TEA$^+$ in the pipette solution and bath solution. Under this condition, an inward current was recorded by depolarizing pulse of 200 ms duration from −40 to 0 mV and inhibited by 1 µM of nifedipine, indicating the current's L-type characteristics (Fig. 4A).

After exposure of the cells to Leo at 50 µM, the current was significantly reduced and returned partially after washout (Fig. 4B). To quantify the magnitude of the effect of Leo on $I_{Ca,L}$, the relative amplitude of $I_{Ca,L}$ to the peak $I_{Ca,L}$ was plotted. Figure 4C shows the change in the relative amplitude of $I_{Ca,L}$ during the bath application of sequentially higher concentration of Leo. Leo at 10, 20, 50, 100 and 200 µM markedly inhibited $I_{Ca,L}$ by 22.93±3.3% ($n=6$, $p<0.05$), 37.91±4.37% ($n=6$, $p<0.01$), 54.48±6.36% ($n=6$, $p<0.01$), 60.27±7.12% ($n=6$, $p<0.01$), and 61.18±7.87% ($n=6$, $p<0.01$), respectively (Fig. 4C). The concentration–response curve of Leo applied in a non-cumulative manner yielded an IC$_{50}$ of 18±5.4 µM ($n=6$).
As demonstrated in Fig. 5, the maximal activation was achieved at 0 mV, and the apparent reversal potential \( E_{\text{rev}} \) was 60 mV. Leo significantly up-shifted the \( I-V \) curve in a concentration dependent manner. The current density at 0 mV was declined from \(-9.11\pm1.01 \text{ pA} \text{ pF}^{-1}\) to \(-5.54\pm0.43 \text{ pA} \text{ pF}^{-1}\) \((n=6, p<0.01)\), and \(-3.46\pm0.67 \text{ pA} \text{ pF}^{-1}\) \((n=6, p<0.01)\) in the presence of Leo at 20 and 100 \( \mu \text{M} \), respectively. However, neither the position of the maximal activation nor the reversal potential was altered. The shape of \( I-V \) curve was partially recovered upon washout of Leo for 5 min \((\text{the current density at 0 mV returned to } -6.66\pm0.63 \text{ pA} \text{ pF}^{-1}, n=6)\).

**Effect of Leo on Channel Recovery of \( I_{\text{Ca,L}} \)**

The time-dependence of recovery from \( I_{\text{Ca,L}} \) inactivation was studied with the paired-pulse protocol illustrated in Fig. 6B. Two pulses \((P_1 \text{ and } P_2)\) from the holding potential \((\text{HP})\) \( -70 \text{ to } 0 \text{ mV} \) were delivered with varying \( P_1-P_2 \) recovery interval, which can be described by the monoexponential equation: 
\[
\frac{I_{\text{Ca,L}}}{I_{\text{max}}}=1-\exp(-t/\tau),
\]
where \( t \) represents the values of the interval, and \( \tau \) represents the time constant of \( I_{\text{Ca,L}} \) recovery from inactivation. The curves in Fig. 6B showed monoexponential function curve, which was fitted to mean data from 6 cells. Leo delayed the recovery time from \( I_{\text{Ca,L}} \) inactivation with right-shifted recovery curve. In the presence of 20 and 100 \( \mu \text{M} \) Leo, the recovery time constant were delayed from 169±22 to 264±25 ms \((n=6, p<0.05)\), and 309±26 ms \((n=6, p<0.05)\).

**Down-Regulation of the L-Type Calcium Channel \( \alpha_{1c} \)**

\( \alpha_{1c} \) subunit by Leo The purpose of this experiment was to determine whether or not mRNA or protein expression level of L-type calcium channel \( \alpha_{1c} \) subunit was regulated by Leo in ventricular myocytes. Semi-quantitative RT-PCR analysis was first used to investigate the effect. As shown in Fig. 7A, the mRNA of \( \alpha_{1c} \) subunit of the L-type calcium channel was expressed in both the control and Leo treated group, and had a lower expression level in the Leo treated group compared to the control using GAPDH as the internal controls \((p<0.05, n=3)\); Fig. 7B). Furthermore, immunoblot analysis also revealed that the amount of \( \alpha_{1c} \) subunit protein was decreased in the heart of neonatal rats treated by Leo. Application of 5, 10,
Leo caused a significant reduction of the $\alpha_{1c}$ subunit protein to 91.5%, 71.8%, 52.1%, 39.4%, 33.8%, 40.8% ($p<0.05, n=3$) compared to control with actin as the internal controls (Figs. 7C, D). Our results suggest that Leo could lead to down-regulation of both mRNA and protein expression of $\alpha_{1c}$ subunit, which may serve as a crucial molecular mechanism underlying the $I_{Ca,L}$ decrease induced by Leo.

**DISCUSSION**

Though Leo was found to reduce the intracellular Ca$^{2+}$ and the myocardial damage provoked in a rat I/R model, no investigation has been reported on the cardiac electrophysiological mechanism of Leo. In the present study, we firstly investigated the ionic mechanism of Leo in rat ventricular myocytes. We found that Leo itself decreases the APD of the rat ventricular myocytes while it had no significant effects on sodium and potassium current. The shortening of APD is mainly attributed to the reduction of $I_{Ca,L}$. The results showed that Leo reduced the peak density of $I_{Ca,L}$, which was accompanied by alteration of the characteristics of inactivation and recovery, and may associate with down-regulation in the mRNA and protein levels of the LTCC $\alpha_{1c}$ subunit.

In our study, we found that though the amplitude of Ca$^{2+}$ channel current was reduced, the $I-V$ relationship curve was not shifted to right or left upon Leo treatment. This suggests that the voltage dependence of the cardiac LTCC was not modified by an interaction with the drug. Moreover, the inactivation kinetics of $I_{Ca,L}$ were changed by Leo. It shifted the steady-state inactivation curve of calcium currents to the
negative potentials, which accelerated the voltage-dependent steady-state inactivation of the calcium currents. Leo also inhibited the recovery from depolarization-induced inactivation. This observation suggests that Leo does not rapidly dissociate from the LTCC. We have also shown here that Leo block the $I_{\text{Ca,L}}$ in a concentration-dependent manner, indicating its blocking effect in rat ventricular myocytes is attributed to the drug itself.

The so-called calcium channel blockers are usually divided into three classes: Dihydropyridine (e.g. nifedipine and its derivative nitrrendipine), Phenylalkylamine (e.g. verapamil and its methoxyderivative D600) and Benzothiazepine (e.g. diltiazem). They are among the most widely prescribed agents for the management of several cardiovascular diseases. Non-dihydropyridine calcium channel blockers are more negatively chronotropic and inotropic than the dihydropyridine subclass, which is important for patients with cardiac dysrhythmias or who need β-blockers. Our previous study in a rat model did not show that Leo had significant negative inotropic effect, suggesting that the action of Leo is more like the dihydropyridines. The electrophysiological profiles of those typical calcium blockers have been intensively studied in the 1980s. All these three type of medicines are able to inhibit LTCC by altering the channel kinetics of inactivation and recovery, among which nifedipine has the slightest effects on channel inactivation and recovery compared to D600 and diltiazem. But when compared to our Leo, the inhibiting effects of those typical blockers are much more stronger. Taking the recovery time as an example, it takes more than 5 times longer (from 97 to 493 ms) for 0.3 $\mu$M nifedipine, 20 times longer for 50 $\mu$M diltiazem and over 100 times longer for 5 $\mu$M D600 to recover, while it only take 1.8 times longer for 100 $\mu$M Leo to recover, suggesting the binding affinity of Leo to inactivated channel state are much weaker than those typical calcium channel blockers. It may, at least in part, explain the weak blocking effect of Leo and it can’t completely inhibit the calcium channel even at high concentration.

Except for the fact that Leo affects channel inactivation and recovery stage, our findings also showed that mRNA and protein of the LTCC α1c subunit in the ventricle was decreased significantly after treatment with Leo. Subunit α1c is the pore region of the channel and it is alone capable of allowing voltage-gated calcium influx. So basically we investigated the short-term effect of Leo on LTCC assayed by the channel kinetics with the electrophysiological technique and the long-term effect on neonatal rat cardiomyocyte by channel protein expression. It has been reported that ischemia results in large increases in cytosolic Ca$^{2+}$ concentration during reperfusion and this rise is generally thought to precede muscle damage. Furthermore, reduction in this increase improves myocardial survival. During ischaemia-reperfusion, the deleterious increase in intracellular Ca$^{2+}$ concentration appears to result from the reverse operation of the Na$^+$/Ca$^{2+}$ exchange mechanism associated with intracellular acidosis and membrane depolarization as well as impaired sarco-endoplasmic reticulum (SR) Ca$^{2+}$-ATPase2 (SERCA2a), which is responsible for SR Ca$^{2+}$ uptake. Factors that diminish the increase in intracellular Ca$^{2+}$ would be expected to attenuate myocardial injury, so downregulation of Ca$^{2+}$ channels should be beneficial. Physiologically, the expression of LTCC mRNA was transiently decreased soon after myocardial infarction followed by recovery towards normal level in a 4-week follow-up period. Our results are in concert with Dr. Gonzalez’s finding that pharmacological preconditioning by diazoxide downregulates cardiac LTCC to exert the cardioprotective effect.

In addition to this calcium channel blocking effect, the previous study had shown that Leo was able to improve the cardiac ischemia along with its other characteristics such as antioxidant and anti-apoptotic effect. In our previous studies, Leo showed protective effects both in doxorubicin and hypoxia induced apoptosis in H9c2 rat ventricular cells. The anti-oxidative and anti-apoptotic efficacy of Leo was $1–10\mu$M in these pathological models. We also found that pretreatment with $10\mu$M Leo reduced intracellular Ca$^{2+}$ overload induced by both doxorubicin and hypoxia. In the present study, Leo was found to inhibit calcium current and downregulate the expression of mRNA and protein of the LTCC α1c subunit at concentration $10\mu$M. Those results suggested that Leo may exert the cardioprotective effects through anti-oxidative and anti-apoptosis at low concentration, while through preventing calcium overloading as well at high concentration.

It is reasonable to pinpoint that Leo exerts its protection on the cardiac myocytes through, at least partly, the ability to inhibit the LTCC. These results may imply the significance of Leo’s potential therapeutic value for some cardiovascular disorders, and it can function as an inhibitor of $I_{\text{Ca,L}}$ in cardiomyocytes. On the other hand, Leo has proved to be a safe compound with the LD$_{50}$ of $5\text{g/kg}$ (data not shown). Even at the concentration as high as 200 $\mu$M, we did not observe significant toxic effects. These pharmacological properties render Leo a promising candidate for clinical application.

In conclusion, this is the first report to detail the ionic evidence that Leo could reduce $I_{\text{Ca,L}}$ and the down-regulated expression of the LTCC α1c subunit might contribute to altered calcium handling in Leo-treated cardiomyocyte. The present results provide a new understanding of the mechanism of cardiomyocyte protection by Leo and may help in the prevention and cure of cardiovascular diseases. However, it should be determined whether Leo has similar effects on human $I_{\text{Ca,L}}$ channels before we move forward to consider the possibility of therapeutic potential of Leo in myocardial ischemia.

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