Dual actions of cilnidipine in human internal thoracic artery: Inhibition of calcium channels and enhancement of endothelial nitric oxide synthase

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Objective: Cilnidipine is a novel, long-action L/N-type dihydropyridine calcium channel blocker that has recently been used for antihypertensive therapy. We investigated the vasorelaxation effect of cilnidipine with regard to its calcium channel blockage and nitric oxide-cyclic guanosine monophosphate-dependent mechanism in human internal thoracic artery.

Methods: Fresh human internal thoracic arteries taken from discarded tissues of patients undergoing coronary artery bypass surgery were studied. Concentration-relaxation curves for cilnidipine in comparison with nifedipine were studied. The expression level of endothelial nitric oxide synthase mRNA was assayed by quantitative real-time polymerase chain reaction, and the phosphorylation of endothelial nitric oxide synthase at Ser¹¹⁷⁷ was determined by Western blotting analysis.

Results: Cilnidipine and nifedipine caused nearly full relaxation in potassium-precontracted internal thoracic artery. Pretreatment with cilnidipine at the clinical plasma concentration significantly depressed the maximal contraction. Endothelium denudation ($47.7\% \pm 7.0\%$, $P < .05$) and inhibition of endothelial nitric oxide synthase ($48.6\% \pm 6.1\%$, $P < .05$) or guanylate cyclase ($41.6\% \pm 3.8\%$, $P < .01$) significantly reduced the cilnidipine-induced endothelium-dependent relaxation ($73.9\% \pm 6.4\%$). Cilnidipine increased the expression of endothelial nitric oxide synthase mRNA by 42.4% ($P < .05$) and enhanced phosphorylation level of endothelial nitric oxide synthase at Ser¹¹⁷⁷ by 37.0% ($P < .05$).

Conclusions: The new generation of calcium channel antagonist cilnidipine relaxes human arteries through calcium channel antagonism and increases production of nitric oxide by enhancement of endothelial nitric oxide synthase. The dual mechanisms of cilnidipine in human arteries demonstrated in this study may prove particularly important in vasorelaxing therapy in cardiovascular diseases. (J Thorac Cardiovasc Surg 2011;141:1063-9)

The use of arterial grafts is common practice in coronary surgery.¹ Because arterial grafts are small-sized arteries, such as the internal thoracic artery (ITA), contraction of the vessel may directly reduce the flow and cause myocardial ischemia.^{2,3}

In its extreme form, vasospasm of the ITA may cause serious ischemic problems that are a long-recognized life-threatening complication after coronary artery bypass grafting (CABG).⁴ Vasospasm is still a recognized problem in current practice even in off-pump CABG.⁵ Because of the serious consequences of vasospasm, the method to prevent and treat spasm in ITA has been an important topic of research.⁶⁻⁹

Calcium (Ca²⁺) channel blockers are commonly used coronary vasodilators to effectively prevent or reverse spasm and associated complications by decreasing vascular resistance and increasing coronary blood flow and myocardial oxygen supply.¹⁰ Dihydropyridine calcium channel blockers have been demonstrated to have an excellent vasorelaxing effect in human ITAs.⁹ However, the clinical benefits of the first-generation dihydropyridine, such as nifedipine (NIF), may be attenuated by the reflex tachyarrhythmia and increased angina.¹¹ Cilnidipine (CIL),^{10,12} a second-generation dihydropyridine, has been used in patients as an antihypertensive vasodilator. CIL exhibits better clinical benefits because of its dual blocking effect on L-type voltage-gated Ca²⁺ channel in vascular smooth muscle and N-type Ca²⁺ channel in sympathetic nerve terminals that supply blood vessels.^{10,13} More recently, it has been demonstrated that CIL may

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Abbreviations and Acronyms

BK	= bradykinin
CABG	= coronary artery bypass grafting
CIL	= cilnidipine
eNOS	= endothelial nitric oxide synthase
ITA	= internal thoracic artery
NE	= norepinephrine
NIF	= nifedipine
NO	= nitric oxide
PCR	= polymerase chain reaction

stimulate release of nitric oxide (NO) from the vascular endothelium.^{14,15} It is possible that this new-generation dihydropyridine derivative may have a particular vasodilatory benefit in antispastic therapy. However, the vasorelaxant effects of CIL and the underlying mechanisms in human arteries are unknown.

The purpose of this study was therefore to assess the efficacy and mechanisms of vasorelaxation of CIL in human arteries. Particular interest was paid to test the hypothesis that the NO-related mechanism of CIL may be due to the enhancement of endothelial nitric oxide synthase (eNOS) in human arteries.

MATERIALS AND METHODS**Organ Bath Technique**

ITA segments ($n = 102$) were collected from 54 patients (42 men and 12 women with a mean age of 64.7 ± 0.8 years) undergoing CABG. Approval to use discarded ITA tissue was given by the institutional review board of Providence St Vincent Hospital. All ITA specimens were collected and placed in the container with oxygenated ice-cold Krebs solution and then delivered to the laboratory. The Krebs solution was composed of (in millimoles/liter) Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2, H_2PO_4^- 1.2, and glucose 11, which was aerated with a gas mixture of 95% O_2 and 5% CO_2 at $37^\circ\text{C} \pm 0.1^\circ\text{C}$ during the period of experiment.

The details of collecting, dissecting, and mounting the ITA rings, and the organ bath technique, have been described.⁹ Briefly, after cautiously dissecting and mounting, the rings were stretched up in progressive steps to determine the length-tension curve for each ring. A computer iterative fitting program (VESTAND 2.1; Yang-Hui He, Princeton, NJ) was used to determine the exponential line, pressure, and internal diameter. When the transmural pressure on the rings reached 100 mm Hg, as determined from their own length-tension curves, the stretch-up procedure was stopped, and the rings were released to 90% of their internal circumference at 100 mm Hg. This degree of passive tension was then maintained throughout the experiment.

After at least 60 minutes of equilibration in the organ bath, 100 mmol/L K^+ was added to the organ bath. The contraction magnitude of the ITA rings was recorded, and the organ bath was then washed with Krebs solution until the resting force was back to the baseline.

RNA Extraction, Polymerase Chain Reaction, and Quantitative Reverse Transcription-Polymerase Chain Reaction

ITA tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, Calif), and total RNA was isolated according to the manufacturer's

specifications. The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm afterward.

Real-time quantitative polymerase chain reaction (PCR) was used to quantify eNOS mRNA expression level. RNA samples of 1 μg were prepared for real-time PCR by random primed RT reaction using random hexamer primers (Promega Corp, Madison, Wis), and the reaction was then diluted 1:10 for PCR quantification, which was conducted in triplicate for increased accuracy. Ten microliters of reaction mixture contained 5 μL TaqMan Universal PCR Master Mix, 0.5 μL Abi eNOS primer and probe mixture, 0.08 μL 18s RNA gene primers and 0.25 μL 18s RNA probe, 2.09 μL diethylpyrocarbonate water, and 2 μL cDNA. The amplification was performed as follows: 2 minutes at 50°C , 10 minutes at 95°C , and then 45 cycles for 15 seconds at 95°C and 60 seconds at 60°C in the ABI PRISM 7900 Sequences Detector Biosystem (PE Applied Biosystems, Foster City, Calif). After PCR was completed, baseline and threshold values were set to optimize the amplification plot, and the data were exported to an Excel spreadsheet (Microsoft Corp, Redmond, Wash). Standard curves were drawn on the basis of the log value of the input RNA versus the critical threshold cycle, under which the critical threshold value could be converted to relative RNA concentration for each sample and normalized by 18s. Values of RNA concentration were finally expressed as fold over control.¹⁶

Western Immunoblotting

ITA samples were homogenized in RIPA Lysis Buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), and the lysates were sonicated in ice for 30 minutes followed by 20 minutes of centrifugation at 20,000g. The supernatant was then collected, and the protein concentration was assayed at 595 nm using the Cary 50 Bio UV-visible spectrophotometer (Varian, Inc, Palo Alto, Calif). After heating the sample for denature at 75°C for 10 minutes, 40 μg protein for each sample was separated by 8% PAGE gel (PAGEgel, Inc, San Diego, Calif) together with the BenchMark pre-stained protein ladder (Invitrogen, Carlsbad, Calif). The proteins were transferred electrophoretically to the transfer membrane (Millipore, Chelmsford, Mass). The membrane was blocked with SuperBlock blocking buffer (Pierce, Rockford, Ill) for 1 hour at room temperature and incubated with primary antibody against eNOS phosphorylated at Ser¹¹⁷⁷ (1:1000) from Upstate (Charlottesville, Va) overnight at 4°C . Equivalent protein on the same lane was confirmed by stripping and reblotting with β -actin (1:20,000; Sigma Aldrich, St Louis, Mo). The secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) at a dilution of 1:10,000 was added the next day. Finally, the membrane was developed with Western Lightning chemiluminescence Reagent Plus detection system (enhanced chemiluminescence reagents; PerkinElmer LAS, Inc, Boston, Mass) and exposed on x-ray films (Kodak, Rochester, NY). Intensity of the protein bands was quantified by Quantity One software (Bio-Rad, Hercules, Calif) and normalized by β -actin and expressed as fold over control.

Protocol

The ITA segments were randomly allocated in various groups to avoid possible influence from patients' demographics.

Relaxation

Cumulative concentration ($-10 \sim -5.5 \log \text{M}$)—relaxation curves for CIL and NIF were established in ITA rings precontracted with K^+ (40 mmol/L, $n = 6$), U_{46619} (10 nM, $n = 6$), or norepinephrine (NE, 10 $\mu\text{mol/L}$, $n = 7$). The concentration of the vasoconstrictors K^+ , U_{46619} , and NE was determined from previous studies. Only 1 concentration-relaxation curve was obtained from each ITA ring. Relaxations to CIL and NIF ($-10 \sim -5.5 \log \text{M}$) were recorded and expressed as a percentage of the vasoconstrictor-induced precontraction. Another group of ITA rings contracted by NE (10 $\mu\text{mol/L}$, $n = 6$) was studied as time control because of its unsustained contraction.

Depression of the Contraction by Pretreatment With Plasma Concentration of Cilnidipine in Internal Thoracic Artery Rings

After equilibration of ITA rings for at least 60 minutes, 100 mmol/L K^+ was added to the organ bath and the contraction force was recorded. The rings were then repeatedly washed with Krebs solution to restore the baseline. To determine whether pretreatment with CIL would alter the contraction response to K^+ , ITA rings were incubated with CIL (20 or 200 nM, or vehicle) for 30 minutes. These concentrations were determined by previous studies in which the plasma CIL concentrations after administration of 10 mg were in the range of 10 to 30 nM.¹⁷ Therefore, we chose 20 nM as the plasma concentration to test the effect in ITA rings. In addition, a 10-fold higher concentration (200 nM) that may be reached after bolus administration was also tested.¹⁷

Cumulative concentration-contraction curves for K^+ (10^{-10} to 100 mmol/L) were then reconstructed. Only 1 concentration-contraction curve was obtained from each ring. The ITA ring taken from the same patient without pretreatment of CIL served as control. Contractions to K^+ (10^{-10} to 100 mmol/L) were recorded and expressed as a percentage of previous 100 mmol/L K^+ -induced precontraction.

Nitric Oxide-cGMP Pathway-Dependent Relaxation

To investigate the role of endothelium denudation, endothelium-derived NO and eNOS, or NO-related cGMP mechanism, CIL (30 nM)-induced relaxation in 40 mmol/L K^+ -induced precontraction was examined in endothelium-denuded ITA rings; the endothelium-intact rings were pretreated with NO synthase inhibitor L-NNA (300 μ mol/L) or guanylate cyclase inhibitor ODQ (3 μ mol/L) for 180 minutes. Endothelium-intact ITA rings taken from the same patient were studied as control. These control rings were also contracted with 40 mmol/L K^+ for 180 minutes. To exclude the effect of the solvent (dimethyl sulfoxide) of CIL (30 nM), the equal amount of dimethyl sulfoxide was added to the bath after 40 mmol/L K^+ .

In the endothelium-denuded ITA rings, the endothelium was removed by gently rubbing the intima with a paper stick.¹⁸

Expression of Endothelial Nitric Oxide Synthase

The artery segment, taken from the same patient undergoing CABG, was divided into 4 rings and randomly assigned to 4 groups as follows: CIL group (incubated with 3 μ mol/L CIL for 4 hours); NE group (incubated with 10 μ mol/L NE for 4 hours); CIL + NE group (incubated with 10 μ mol/L NE for 30 minutes followed by 3 μ mol/L CIL for 4 hours); and control group (incubated with vehicle). All arteries were then flash-frozen in liquid nitrogen and stored at -80°C for further study.

RNA in ITA tissue was extracted as described. Then, 1 μ g RNA samples were prepared for reverse transcription, and 2 μ g cDNA were used to perform real-time PCR to quantify eNOS mRNA expression level.

Phosphorylation of Endothelial Nitric Oxide Synthase at Ser¹¹⁷⁷

The artery segment, taken from the same patient undergoing CABG, was divided into 3 rings and randomly assigned to 3 groups as follows: CIL group (incubated with 10 μ mol/L NE for 15 minutes followed by 3 μ mol/L CIL for 10 minutes); bradykinin (BK) group (incubated with 10 μ mol/L NE for 15 minutes followed by 50 nM BK for 5 minutes); and control group (incubated with 10 μ mol/L NE for 15 minutes followed by incubation with vehicle of CIL). BK was used to stimulate the eNOS expression because it is a classic endothelium-dependent vasodilator known to increase eNOS and NO release. All arteries were then flash-frozen in liquid nitrogen and stored at -80°C for further study.

As mentioned earlier, protein in ITA tissue was extracted with RIPA buffer. Then, 40 μ g protein samples were separated by 8% PAGE gel and transferred electrophoretically to the transfer membrane. After being

FIGURE 1. Mean concentration (log M)/relaxation (%) curves for CIL and NIF in human ITA rings precontracted with (A) potassium chloride (K^+ , 40 mmol/L, $n = 6$), (B) U_{46619} (10 nM, $n = 6$), and (C) NE (30 μ mol/L, $n = 7$). Values are expressed as mean $\pm$ standard error (SE). $P > .05$, compared with each other (1-way analysis of variance followed by Bonferroni's multiple comparison test). CIL, Cilnidipine; NIF, nifedipine; NE, norepinephrine.

incubated with primary and secondary antibodies, the membrane was exposed on x-ray films. Densitometry was then performed for the band to quantify relatively eNOS phosphorylation level.

FIGURE 2. Mean concentration (log M)/contraction (%) curves for potassium chloride (K^+ , 10 ~ 100 mmol/L) in the human ITA rings pretreatment with CIL (-6.7 and -7.7 logM). Values are expressed as mean $\pm$ SE, $n = 6$. * $P < .05$, ** $P < .01$, compared with control. CIL, Cilnidipine.

Materials

BK, L-NNA, ODQ, NIF, and NE were purchased from Sigma-Aldrich (St Louis, Mo). CIL was a gift from Fujirebio (Tokyo, Japan). U_{46619} was purchased from Cayman Chemical Company (Philadelphia, Pa); stock solutions were held at -20°C . Stock solutions of CIL and NIF at concentration of 0.1 mol/L were diluted in dimethyl sulfoxide and kept frozen until required.

Statistical Analyses

The relaxation effect of CIL and NIF was recorded and expressed as a percentage of the vasoconstrictor-induced precontraction. Statistical comparisons of the maximal percentage relaxation, expression level of eNOS mRNA, and phosphorylation of eNOS at Ser¹¹⁷⁷ under different treatments were performed by 1-way analysis of variance followed by Bonferroni's comparison test. Statistical comparisons of cumulative contraction force and cumulative percentage of relaxation under different treatments were performed by 2-way analysis of variance followed by Bonferroni's test.

The efficacy of both vasoconstrictors and vasodilators was expressed as EC_{50} , the effective concentration causing 50% of maximal contraction or relaxation. The EC_{50} was determined from each concentration-contraction or concentration-relaxation curve by a logistic curve-fitting equation: $E = MA^p / (A^p + K^p)$, where E is response, M is maximal contraction or relaxation, A is concentration, K is EC_{50} concentration, and p is the slope parameter. A computerized program was used for the curve fitting. EC_{50} values between different groups were analyzed by unpaired t test. All values were expressed as mean $\pm$ standard error.

RESULTS

Resting Parameters of Internal Thoracic Artery Rings in Contraction or Relaxation Studies

The internal diameter of the 73 vessels at an equivalent transmural pressure of 100 mm Hg (D_{100}) was 2.45 ± 0.06 mm, as determined from the computerized normalization procedure. The resting transmural pressure was 74.7 ± 0.7 mm Hg at 90% of D_{100} . The resting force of the ITA rings was 3.2 ± 0.2 g.

A

B

C

FIGURE 3. Time (minutes)/relaxation (%) curves for CIL (30 nM) in endothelium-intact ($n = 7$) and denuded ($n = 7$) ITA rings with or without ODQ ($3 \mu\text{mol/L}$, $n = 8$) or L-NNA ($300 \mu\text{mol/L}$, $n = 8$) precontracted with 40 mmol/L potassium chloride (K^+). Endothelium-intact ITA rings taken from the same patient were studied as control. These control rings were also contracted with 40 mmol/L K^+ for 180 minutes. To exclude the effect of the solvent (dimethyl sulfoxide) of CIL (30 nM), the equal amount of dimethyl sulfoxide was added in the bath of these control rings after 40 mmol/L K^+ . Values are expressed as mean $\pm$ SE. * $P < .05$, ** $P < .01$, compared with endothelium-intact control group.

FIGURE 4. Expression of eNOS mRNA in human ITA rings treated with 3 $\mu\text{mol/L}$ CIL ($n = 6$), 10 $\mu\text{mol/L}$ NE ($n = 6$), 3 $\mu\text{mol/L}$ CIL, and 10 $\mu\text{mol/L}$ NE ($n = 5$), for 4 hours, assayed by quantitative real-time PCR. The level of eNOS expression was normalized by 18s and expressed as fold over control. Values are expressed as mean $\pm$ SE, $n = 5, 6$. $*P < .05$, compared with control. CIL, Cilnidipine; NIF, nifedipine; NE, norepinephrine; eNOS, endothelial nitric oxide synthase.

Similar Relaxation Effect of Cilnidipine and Nifedipine in Human Internal Thoracic Artery Rings Precontracted With K^+ , U_{46619} , and Norepinephrine

CIL and NIF-induced relaxation were studied in human ITA rings precontracted with different vasoconstrictors: K^+ (40 mmol/L, $n = 6$), U_{46619} (10 nM, $n = 6$), and NE (10 $\mu\text{mol/L}$, $n = 7$). CIL and NIF caused nearly full relaxation ($94.7\% \pm 2.6\%$ vs $100\% \pm 0\%$) with similar EC_{50}s ($-8.90 \pm 0.90 \log \text{M}$ vs $-8.46 \pm 1.08 \log \text{M}$) in K^+ -precontracted ITA rings and less relaxation ($82.4\% \pm 8.1\%$ vs $84.5\% \pm 9.9\%$; EC_{50}s : $-7.50 \pm 0.44 \log \text{M}$ vs $-7.43 \pm 0.41 \log \text{M}$, $P = .19$ for CIL and $P = .39$ for NIF, compared with K^+ -induced precontraction) in U_{46619} -precontracted ITA rings. In ITA rings precontracted with NE, because the contraction was unstable, the relaxation was adjusted by the time control in the ring, which was contracted by NE, but no vasodilators were tested. The relaxation was significantly less in NE precontraction ($36.3\% \pm 6.2\%$ vs $40.4\% \pm 3.9\%$; EC_{50}s : $-6.55 \pm 0.69 \log \text{M}$ vs $-6.68 \pm 0.47 \log \text{M}$, $P = .06$ for CIL and $P = .14$ for NIF, compared with K^+ -induced precontraction) (Figure 1).

Depression of the Contraction by Pretreatment With Plasma Concentration of Cilnidipine in Internal Thoracic Artery Rings

After incubating CIL (20, 200 nM) for 30 minutes, cumulative concentration-contraction curves to K^+ in ITA rings with and without CIL incubation were established, and the contraction was expressed as percentage of the contraction to 100 mmol/L K^+ (Figure 2). Pretreatment with 20 nM CIL slightly altered K^+ -induced contraction force ($88.7\% \pm 10.2\%$ vs $100.8\% \pm 11.0\%$, $P > .05$) and the EC_{50}s ($29.3 \pm 18.7 \text{ mmol/L}$ vs $36.4 \pm 12.3 \text{ mmol/L}$, $P > .05$).

A

B

FIGURE 5. eNOS and phosphorylation of eNOS at Ser¹¹⁷⁷ in human ITA stimulated with 10 $\mu\text{mol/L}$ NE for 15 minutes followed by 10-minute incubation with 3 $\mu\text{mol/L}$ CIL ($n = 6$) or 5-minute incubation with 50 nM BK ($n = 6$), examined by Western blot analysis. Incubation with vehicle was used as control. A, Representative Western blot abundance depicting eNOS and the phosphorylation of eNOS at Ser¹¹⁷⁷. Photographs were modified by Photoshop software (Adobe Systems Inc, San Jose, CA) with the same brightness and contrast. B, Optical density of eNOS phosphorylated at Ser¹¹⁷⁷, normalized by β -actin and expressed as fold over control. Values are expressed as mean $\pm$ SE. $*P < .05$, $**P < .01$, compared with control. peNOS, Phosphorylation of eNOS; eNOS, endothelial nitric oxide synthase; CIL, cilnidipine; BK, bradykinin.

Further, CIL at the concentration of 200 nM significantly reduced the magnitude of the contraction induced by K^+ at concentration from 40 to 100 mmol/L and right-shifted the curve compared with the control group (EC_{50} : $95.0 \pm 21.5 \text{ mmol/L}$ vs $36.4 \pm 12.3 \text{ mmol/L}$, $P < .01$). The maximal contraction force induced by K^+ was 59.2% of the control ($59.7\% \pm 6.7\%$ vs $100.8\% \pm 11.0\%$, $P < .01$).

Cilnidipine-Induced Relaxation Is Altered by Endothelium-Denudation, L-NNA, or ODO: The Role of the Nitric Oxide-cGMP Pathway

In 40 mmol/L K^+ -induced contraction, CIL-induced relaxation was significantly attenuated by denudation of endothelium at 180 minutes ($47.7\% \pm 7.0\%$ vs $73.9\% \pm 6.4\%$, $P < .05$, Figure 3, A). A similar effect was seen in endothelium-intact ITA rings pretreated with 300 $\mu\text{mol/L}$ L-NNA ($48.6\% \pm 6.1\%$ vs $73.9\% \pm 6.4\%$, $P < .05$, Figure 3, B). When the endothelium-intact ITA rings were incubated with 3 $\mu\text{mol/L}$ ODO (guanylate cyclase inhibitor), the magnitude of CIL-induced relaxation was depressed even more dramatically during the whole observation period ($41.6\% \pm 3.8\%$ vs $73.9\% \pm 6.4\%$, $P < .01$, Figure 3, C).

Expression Level of Endothelial Nitric Oxide Synthase mRNA in Human Internal Thoracic Artery Tissue

The expression level of eNOS mRNA in ITA tissue stimulated with 10 $\mu\text{mol/L}$ NE for 30 minutes followed by 3 $\mu\text{mol/L}$ CIL incubation for 4 hours was increased obviously by 42.4% (1.0 ± 0.1 vs 1.4 ± 0.2 , $P < .05$) (Figure 4). However, treatment with CIL or NE had no significant effect on the expression level of eNOS mRNA.

Enhanced Phosphorylation of Endothelial Nitric Oxide Synthase at Ser¹¹⁷⁷ in Human Internal Thoracic Artery Tissue

In human ITA stimulated by 10 $\mu\text{mol/L}$ NE for 15 minutes followed by the incubation with 3 $\mu\text{mol/L}$ CIL for 10 minutes or 50 nM BK for 5 minutes, the phosphorylation of eNOS at Ser¹¹⁷⁷ increased dramatically (Figure 5, A). In contrast, there was no significant change in eNOS (Figure 5, A). The optical density value for CIL-treated tissues was increased by 37.0% ($P < .05$) in comparison with control (Figure 5, B).

DISCUSSION

The present study found that (1) CIL is an effective vasodilator in the human ITA, and this provides important information for clinical use of this new generation of dihydropyridine; (2) CIL has dual mechanisms in the relaxation effect, including calcium channel inhibition and endothelium-dependent mechanism; and (3) unlike traditional calcium antagonists, CIL enhances eNOS expression, which may open a new direction in the development of new vasodilators.

It is now well known that calcium is the principal mediator involved in vasomotion¹⁹ and that influx of Ca^{2+} through the L-type calcium channel triggers cell contraction and is inhibited by calcium channel blockers.^{20,21}

Available oral calcium channel blockers as antihypertensive drugs are classified into 4 categories or generations.^{22,23} CIL is a long-acting N-type calcium channel blocker, and it is considered a fourth-generation calcium channel blocker that suppresses the activity of the sympathetic nervous system via the N-type calcium channel.²³ Therefore, compared with other calcium channel blockers, apart from its ability to lower blood pressure, the particular benefits of CIL are significant suppression of increases in heart rate, epinephrine, and NE.²⁴ These benefits may make CIL clinically useful in patients with coronary artery disease and combined hypertension, which is one of the major risk factors for coronary artery disease.

It is suggested that CIL^{14,15} may release NO from vascular endothelium in animal vessels. However, this has not been demonstrated in human vessels. In the present study, the NO-stimulating effect of CIL is demonstrated by the endothelium-dependent relaxation induced by CIL, as well

as the reduction of the relaxation by eNOS inhibitor L-NNA and the soluble guanylate cyclase inhibitor ODC.

The molecular studies also support the hypothesis that CIL up-regulates eNOS mRNA and protein production. When the vessel was incubated with CIL and NE, the increase of eNOS mRNA was significant, although the effect of CIL alone was weak. In the study on the eNOS protein, the increase of the protein was as significant as the classic eNOS stimulant BK that clearly shows the enhancement of eNOS by CIL. This is particularly important when there is endothelial dysfunction, as in patients with coronary artery disease. There is often associated endothelial dysfunction in various vasculatures, and frequently associated diseases, such as diabetes and hypertension, are also accompanied by endothelial dysfunction.

These studies clearly demonstrated the dual mechanisms of CIL in the human vessels used as coronary artery bypass grafts and therefore support the use of this fourth-generation calcium channel blocker for coronary artery disease because this drug may relax the coronary artery by a direct relaxing effect and enhancement of the NO-related endothelial function. Endothelial dysfunction is an early sign of coronary artery disease.^{25,26}

Finally, unlike NIF, which can be used only orally, CIL can be used orally or intravenously,²⁷ and therefore can be used topically during surgery as a topical antispastic solution.

CONCLUSIONS

The new generation of calcium channel antagonist CIL relaxes human arteries through calcium channel antagonism and increased production of NO by enhancement of eNOS. The dual mechanisms of CIL in human arteries demonstrated in this study may prove particularly important in vasorelaxing therapy in cardiovascular diseases.

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