# 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 $Ser^{1177}$ 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 $Ser^{1177}$ 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 ($Ca^{2+}$) 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 $Ca^{2+}$ channel in vascular smooth muscle and N-type $Ca^{2+}$ 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°C ± 0.1°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 20,000*g*. 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 prestained 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 $Ser^{1177}$ (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 ~ –5.5 log 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 μmol/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 ~ –5.5 log M) were recorded and expressed as a percentage of the vasoconstrictor-induced precontraction. Another group of ITA rings contracted by NE (10 μmol/L, n = 6) was studied as time control because of its unsustained contraction.