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Cilnidipine, a slow-acting Ca^{2+} channel blocker, induces relaxation in porcine coronary artery: role of endothelial nitric oxide and $[Ca^{2+}]_i$

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1 Cilnidipine is a dual blocker of L-type voltage-gated Ca^{2+} channels in vascular smooth muscle and N-type Ca^{2+} channels in sympathetic nerve terminals that supply blood vessels. However, the clinical benefits of cilnidipine and underlying mechanisms are incompletely understood. This study was designed to compare the time course of relaxant responses to cilnidipine and nifedipine, and to examine the role of endothelial NO and $[Ca^{2+}]_i$ in the vasorelaxation.

2 Porcine left circumflex coronary arteries were isolated and isometric tension was measured with Grass force transducers. Endothelial $[Ca^{2+}]_i$ in intact arteries was determined by a calcium fluorescence imaging technique. The free radical scavenging capacity was also assayed.

3 Cilnidipine and nifedipine induced concentration-dependent relaxations in high KCl-precontracted artery rings, while the former-induced relaxation was slower as compared to the latter. Treatment with L-NAME or ODQ reduced relaxations to cilnidipine or nifedipine to the same extent as in rings without endothelium. Indomethacin or ω-conotoxin had no effects. L-Arginine antagonized the effect of L-NAME on cilnidipine-induced relaxations. Cilnidipine did not affect sodium nitroprusside-induced relaxation in rings with and without endothelium.

4 Cilnidipine and nifedipine caused extracellular Ca^{2+}-dependent increases in endothelial $[Ca^{2+}]_i$ in intact arteries and cilnidipine's action had a slower onset, similar to that of cilnidipine-induced relaxation.

5 Neither cilnidipine nor nifedipine exhibited a free radical scavenging property.

6 The present results demonstrate that cilnidipine can produce endothelium-dependent relaxation in porcine coronary arteries *in vitro* in addition to blocking Ca^{2+} channels. Like short-acting nifedipine, cilnidipine-dependent relaxation, albeit to a slower onset, is partly mediated by endothelial NO but not by prostacyclin. The increased release or bioavailability of NO may causally result from elevated endothelial $[Ca^{2+}]_i$ in arteries. The Ca^{2+} channel-independent effect suggests the usefulness of cilnidipine in the treatment of cardiovascular diseases associated with diminished NO release, such as atherosclerosis.

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Abbreviations: DHP, dihydropyridine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDHF, endothelium-derived hyperpolarizing factor; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one

Introduction

Dihydropyridine (DHP) Ca^{2+} antagonists, including nifedipine, are frequently used in the treatment of hypertension, stable angina pectoris and cerebrovascular disease by blocking L-type voltage-gated Ca^{2+} channels. These therapeutic agents relax coronary arteries, cause peripheral vasodilatation, and decrease left ventricular contraction, thereby reducing the myocardial oxygen demand. The clinical studies and meta-analysis, however, have questioned the long-term safety of nifedipine regimen that actually increases the risk of myocardial infarction and mortality in patients with coronary artery disease (Furberg *et al*., 1995; Psaty *et al*., 1995; Stason *et al*., 1999). The deleterious mechanism for this fatal adverse effect of nifedipine is unclear. Rapid hypotensive action of short-acting DHPs causes an elevation in vascular sympathetic tone and reflex tachycardia. The increased release of noradrenaline may represent a coronary risk factor in hypertensive patients (Julius, 1993).

In contrast to nifedipine, a short-acting first generation DHP that exclusively blocks L-type Ca^{2+} channels, cilnidipine is a new, long-acting second generation DHP that blocks both L- and N-type Ca^{2+} channels. Cilnidipine is a slow-onset vasodilator and antihypertensive drug as revealed in clinical and animal studies (Yoshimoto *et al*., 1991; Tominaga *et al*.,

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1997). Cilnidipine blocks L-type Ca^{2+} channels in vascular smooth muscle at concentrations that do not inhibit protein kinase C (Lohn *et al*., 2002), while nifedipine is a potent inhibitor of protein kinase C (Hempel *et al*., 1999). Besides, cilnidipine also inhibits T-type Ca^{2+} currents in guinea-pig ventricular myocytes (Takeda *et al*., 2004).

Cilnidipine attenuates vascular sympathetic neurotransmission (Hosono *et al*., 1995) probably by blocking the N-type Ca^{2+} channels (Fujii *et al*., 1997). With this unique dual L/N-type blocking property, cilnidipine may have clinical advantages over nifedipine with respect to cardiovascular protection. Indeed, cilnidipine effectively lowers blood pressure with much less influence on heart rate and sympathetic nerve activity than nifedipine in hypertensive patients (Minami *et al*., 1998; 2000). Cilnidipine is also demonstrated to reduce blood pressure in animal studies (Nakajima *et al*., 2002; Varagic *et al*., 2002).

The endothelium regulates the vascular tone and blood pressure. Endothelial dysfunction characterized by impaired endothelium-dependent vasodilatation has been described in both human and animal hypertension (Mombouli & Vanhoutte, 1999). Some DHPs exhibit Ca^{2+} channel-independent vascular effects leading to improved endothelial function (Taddei *et al*., 1997; Krenek *et al*., 2001). Recent *in vitro* studies have shown that nifedipine enhances NO release *via* the upregulation of eNOS expression (Ding & Vaziri, 2000) and possesses antioxidant or free radical scavenging effects (Lesnik *et al*., 1997).

As compared with nifedipine, it is, however, unknown whether cilnidipine exerts similar benefits on the endothelium by releasing relaxing substances. The present study tested the hypothesis that cilnidipine produces endothelium-dependent relaxation in porcine coronary arteries *in vitro* in addition to its known Ca^{2+} channel-blocking property. Specifically, this study examined (i) whether cilnidipine induces endothelium-dependent relaxation and what endothelium-derived vasoactive factors are involved, (ii) whether cilnidipine stimulates an increase in endothelial $[Ca^{2+}]_i$ *in situ* in freshly isolated arteries, and (iii) whether cilnidipine possesses a free radical-scavenging activity. For comparison, we studied the arterial effects of nifedipine, the clinically relevant prototype of DHP Ca^{2+} antagonists in the same preparations.

Methods

All experiments were in accordance with institutional guidelines. This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Vessel preparation

Fresh pig hearts were obtained from a local slaughterhouse in Hong Kong. The hearts were placed on a dissecting plate filled with ice-cold Krebs solution. Krebs solution contained (in mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose. After fatty connective tissues were removed, the left circumflex coronary artery was cut into several rings (3 mm length). Rings were suspended in 10-ml organ baths and bathing solution was oxygenated with 95% O_2–5% CO_2 and maintained at 37°C (pH: 7.3–7.5). Each ring was stretched to an optimal tension of 15 mN and then allowed to equilibrate for 90 min before the start of the experiment. In some rings, the endothelial layer was disrupted by gently rubbing the luminal surface with forceps tip. Removal of a functional endothelium was accepted if no relaxation to 50 nM bradykinin was obtained. Each experiment was carried out on rings prepared from different pigs.

Force measurement

At 30 min after setting up in organ baths, each ring was initially contracted by 30 nM U46619 and subsequently relaxed by 50 nM bradykinin to confirm the presence of a functional endothelium. Rings were then washed in pre-warmed Krebs solution until baseline tone was returned. High KCl solution was prepared by substituting an equivalent amount of NaCl with KCl in order to maintain the same ionic strength.

The first series of experiments examined the relaxant effects of different concentrations of cilnidipine (3–100 nM) or nifedipine (3–100 nM) in 60 mM KCl-contracted rings with endothelium. The relaxant responses were allowed to reach a steady-state level. The second series of experiments used 30 mM KCl solution, an ionic condition that eliminates the involvement of endothelium-derived hyperpolarizing factors (EDHF) in porcine coronary arteries since a small relaxation to bradykinin in 30 mM KCl-contracted rings was abolished by L-NAME (a NOS inhibitor). Since reduced relaxations to both DHP agents in the absence of endothelium were obtained, the role of endothelium-derived NO or prostacyclin was studied. Rings with endothelium were incubated for 30 min with L-NAME (100 μM), ODQ (guanylate cyclase inhibitor, 3 μM), or indomethacin (inhibitor of prostacyclin biosynthesis, 3 μM) prior to contraction by 30 mM KCl. The effect of ω-conotoxin (a N-type Ca^{2+} channel blocker, 100 nM) was also tested. Once a steady tone was obtained, cilnidipine or nifedipine (each at 30 nM) was added to induce relaxation. In order to test whether treatment with L-arginine could antagonize the effect of L-NAME, rings with endothelium were exposed to 500 μM L-arginine 10 min before the addition of L-NAME. The third group of experiments examined the relaxant effects of cilnidipine and nifedipine in L-NAME-treated rings without endothelium. The role of the endothelium in DHP-induced relaxation was also examined in rings contracted by 60 mM KCl. The presence of endothelium did not affect high KCl-induced vessel tone (30 mM KCl contraction: 35.9 ± 2.8 mN with endothelium and 35.5 ± 2.0 mN without endothelium, $P > 0.05$; 60 mM KCl contraction: 41.7 ± 2.9 mN with endothelium and 41.3 ± 2.6 mN without endothelium, $P > 0.05$).

Finally, effects of cilnidipine (10 nM, 30-min exposure) were tested on endothelium-independent relaxation to NO donor, sodium nitroprusside (200 μM), in the presence and absence of endothelium.

In situ *endothelial $[Ca^{2+}]_i$ imaging*

Porcine left circumflex coronary artery rings were labeled fluorescently by the incubation with 10 μM Fura-2 AM, 0.025% pluronic F-127, and 1 mM probenacid (to prevent Fura-2 secretion) in Krebs solution for 1 h at 22°C. Extracellular Fura-2 AM was washed in Krebs solution. The endothelial cells were then perfused for 20 min with Krebs solution at 2 ml min^{-1} (37°C) to permit intracellular esterases to cleave intracellular Fura-2 AM into active Fura-2.

The basic setup for endothelial $[Ca^{2+}]_i$ imaging was modified as first described by Huang *et al.* (2000). Briefly, after the Fura-2 loading, the rings were longitudinally cut open and pinned down onto a block of silicone elastomer, which was fixed onto a base plate of the custom-made flow chamber. The base plate was then covered with a gasket and cover glass (24×32 mm), and affixed by screws. There was a 1-mm gap between the vessel lumen and cover glass for fluid flow passage. After mounting, the flow chamber was placed on an inverted microscope and perfused with Krebs solution at 37°C at 2 ml min^{-1} maintained by a six-channel perfusion pump. The specimen was illuminated on the stage of the IX70 Olympus microscope, fitted with an $\times 20$ Olympus water immersion objective by using a Polychrome IV light source. Fura-2 loaded vascular tissues were alternately excited at 340 and 380 nm, and images of the respective 510-nm emissions were collected in 1-s intervals using MetaFluor v4.6 software (Universal Imaging Corp., Downingtown, PA, U.S.A.). The emitted light was transmitted to the collecting device and then to a cooled charge coupled device (CCD) camera. Illumination through the Polychrome IV light source and acquisition by the CCD camera were controlled by MetaFluor software v4.6. Video frames containing images of cell fluorescence were digitized at a resolution of 512 horizontal $\times$ 480 vertical pixels. Imaging analysis was performed with a MetaFluor imaging system (Universal Imaging Corp.). After background subtraction, the fluorescence ratio (F340/F380) was obtained by dividing, pixel by pixel, the 340 nm image by the 380 nm image. Changes in this ratio reflected changes in $[Ca^{2+}]_i$ to eliminate potential artifacts caused by variations in cell thickness, intracellular dye distribution, or photobleaching.

Free radical scavenging assay

Possible free radical scavenging effects of DHPs were examined as described previously (Blois, 1958). α-Tocopherol (vitamin E) was used as a reference antioxidant. In brief, 0.5 ml of methanol containing different concentrations (10–100 nM) of DHPs was mixed in a test tube with 2.5 ml of methanol containing 75 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is a stable free radical with a deep purple color in ethalonic solution and a typical absorbance at 517 nm, but becomes pale yellow when trapped by an antioxidant. The reaction mixture was kept in dark at room temperature for 90 min and the absorbance at 517 nm was thereafter measured. The free radical scavenging activity was approximated using the following equation:

$$\text{DPPH scavenging activity}(\%) = [A_a - (A_b - A_c)]/A_a \times 100$$

where A_a is the absorbance of the incubation DPPH solution without DHP, A_b is the absorbance of the incubation mixture containing DPPH and DHP, and A_c is the absorbance of the blank solution (methanol) with DPPH.

Chemicals

Bradykinin, U46619, L-NAME, ODQ, L-arginine, indomethacin, nifedipine, ω-conotoxin, sodium nitroprusside, and DPPH were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Cilnidipine was a gift from Fujirebio (Tokyo, Japan). Stock solution was prepared in DMSO for DHPs and kept at −20°C. Desired dilution was made before experiments. DMSO (0.1%, v v^{-1}) did not cause vasorelaxation.

Data analysis

Results are mean ± s.e.m. and n refers to the number of pigs. Several rings prepared from the same artery were studied in parallel. Time-dependent relaxant effects of DHPs were investigated after various pharmacological interventions. The relaxation was expressed as percentage reduction in high KCl-induced tone and relaxation curves were analyzed and time ($t_{1/2}$) required for producing 50% of the maximal relaxation was calculated using Graphpad software (Version 3.0). Time-dependent relaxation curves were analyzed by two-way ANOVA followed by Bonferroni post-tests. P-values less than 0.05 were regarded as statistically significant.

Results

Relaxant responses

Both cilnidipine and nifedipine relaxed 60 mM KCl-contracted rings with endothelium in a concentration-dependent manner (Figure 1a and c). There was little difference in relaxing potency of both blockers as determined by steady-state relaxation (Figure 1b and d). The onset of relaxation was, however, much faster for nifedipine than cilnidipine at each concentration tested (Figure 1a and c). Nifedipine at 100 nM induced a rapid relaxation, which reached a plateau within 10 min after drug application (Figure 1c). In contrast, cilnidipine at the same concentration induced slowly developing relaxation and the steady-state maximum effect was obtained 80 min after drug application (Figure 1a). Figure 1e shows differences in the $t_{1/2}$ values for relaxations induced by both DHP agents.

Role of the endothelium

In 30 mM KCl-contracted rings with endothelium, cilnidipine at 30 nM caused relaxation and the peak response was obtained after 120 min and this relaxation was attenuated upon removal of the endothelium (Figure 2a). Likewise, nifedipine at 30 nM induced less relaxation in rings without endothelium than in rings with endothelium (Figure 3a).

Effect of inhibitors of NO-dependent relaxation

Treatment of rings with endothelium by L-NAME (Figure 2b) or ODQ (Figure 2c) attenuated cilnidipine-induced relaxation. L-NAME and ODQ reduced cilnidipine-dependent relaxation to a similar extent as seen in rings without endothelium. Preincubation with L-arginine, the precursor of NO biosynthesis, abolished the inhibitory effect of L-NAME (Figure 2b and d). Likewise, L-NAME and ODQ reduced relaxation induced by nifedipine (30 nM) and L-arginine antagonized the effect of L-NAME (Figure 3b and c). In contrast, L-NAME did not affect relaxations to cilnidipine or nifedipine in rings without endothelium ($n = 5$, data not shown). Relaxation induced by cilnidipine or nifedipine in 60 mM KCl-contracted rings was the same in the presence and absence of endothelium (Figure 4). Bradykinin (50 nM) induced only a small relaxation

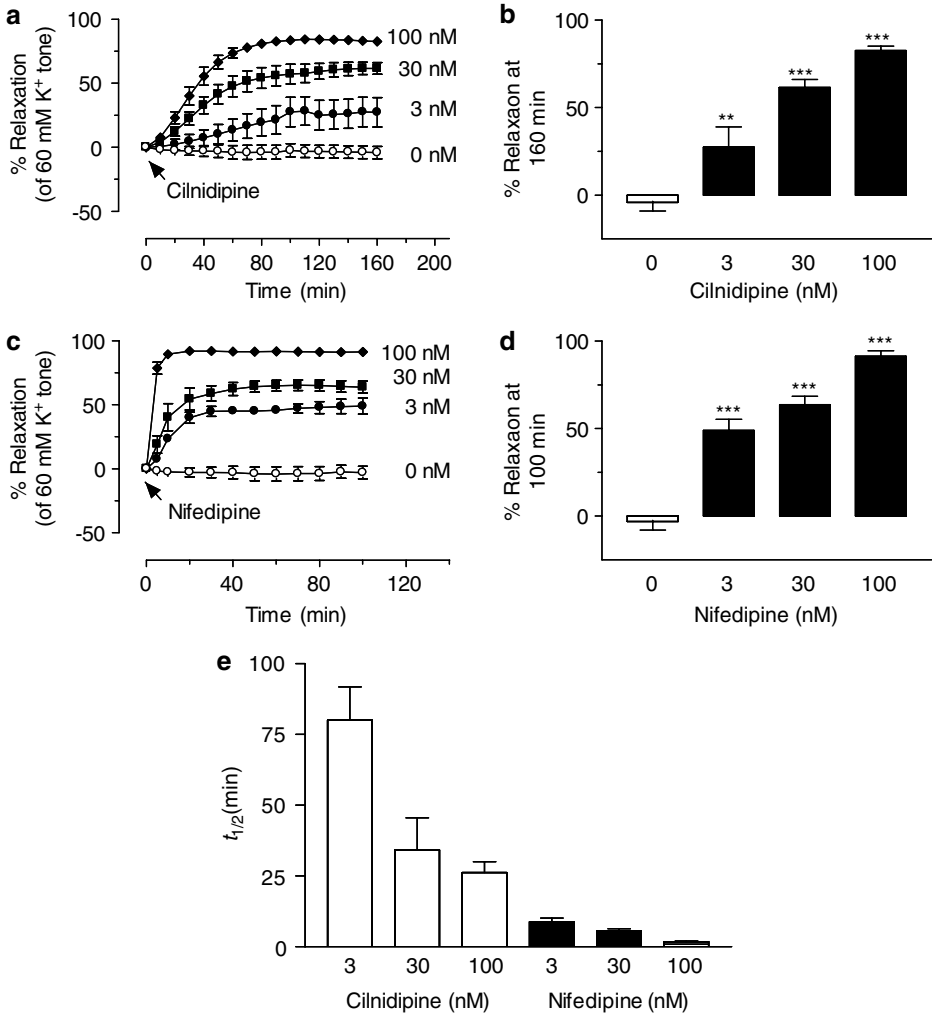

Figure 1 Concentration-dependent relaxant responses to cilnidipine (a) or nifedipine (c) in 60 mM KCl-contracted porcine coronary artery rings with endothelium. The percentage relaxation recorded at 160 min (b) after application of cilnidipine (3–100 nM) or at 100 min (d) after application of nifedipine (3–100 nM). (e) The time taken for cilnidipine or nifedipine to produce half ($t_{1/2}$) of the maximal relaxant effect. Results are mean ± s.e.m. of 6–7 experiments. Statistical difference is indicated by $^{**}P<0.01$ or $^{***}P<0.001$ from controls.

(21.2 ± 4.6%) in 30 mM KCl-contracted rings with endothelium, which was abolished by 100 μM L-NAME and bradykinin did not relax 60 mM KCl-contracted rings with endothelium ($n=4$).

Effect of indomethacin and ω-conotoxin

Indomethacin did not modify relaxations to cilnidipine or nifedipine ($n=5$), thus ruling out the involvement of relaxing prostanoids in the endothelium-dependent effects for both DHPs. ω-Conotoxin (100 nM) did not affect 30 mM KCl-induced tone nor cilnidipine-induced relaxation ($n=4$, data not shown).

Effect of cilnidipine on sodium nitroprusside-induced relaxation

Cilnidipine (10 nM, 30-min incubation) inhibited 30 mM KCl-induced contraction of rings with endothelium (35.8 ± 2.8 mN in control and 21.0 ± 3.2 mN in cilnidipine). However, cilnidipine did not affect relaxations to 200 μM sodium nitroprusside in the presence and absence of endothelium (Figure 5).

Effects on endothelial $[Ca^{2+}]_i$ in isolated endothelium-intact coronary arteries

Fluorescence images from individual living endothelial cells in intact cut-open rings were clearly visible and signals were absent upon removal of the endothelium. An increase in $[Ca^{2+}]_i$ was induced by bradykinin only in endothelium-intact arteries (Figure 6a). Figure 6b shows that perfusion of 30 nM cilnidipine caused a slow increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was long-lasting (80 min) and completely abolished in the absence of extracellular Ca^{2+} (plus 300 μM Na_2-EGTA). Likewise, addition of 30 nM nifedipine induced a similar but faster effect in raising endothelial $[Ca^{2+}]_i$, which was also dependent on the presence of extracellular Ca^{2+} ions (Figure 6c).

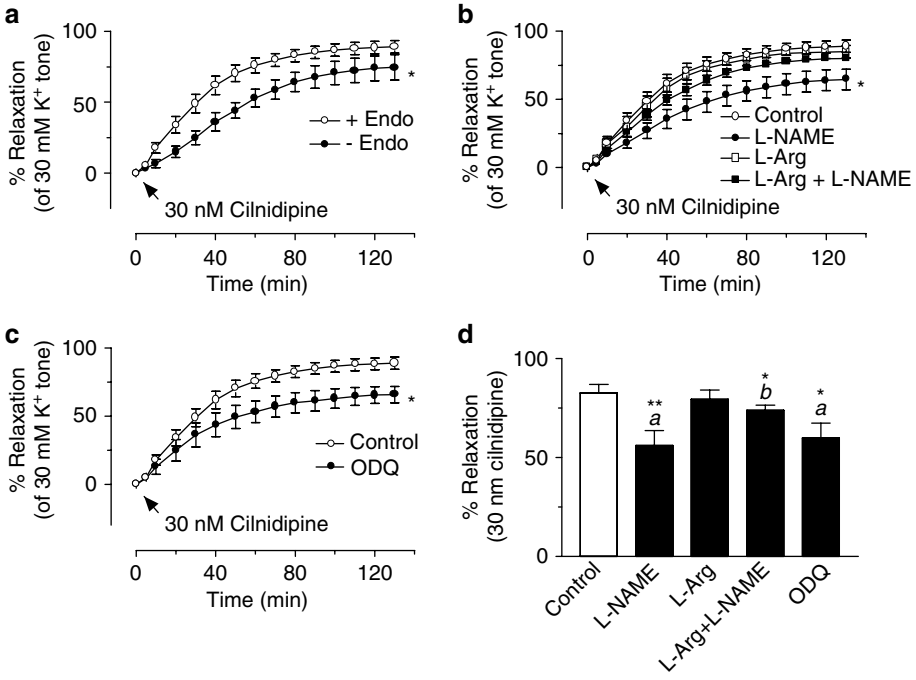

Figure 2 (a) Reduced relaxation to 30 nM cilnidipine in rings without endothelium compared to rings with endothelium. Attenuated cilnidipine-induced relaxation following treatment with L-NAME (b) or ODQ (c) in rings with endothelium. Treatment with L-arginine reversed the effect of L-NAME (b). The relaxant effect recorded at 30 min after application of cilnidipine (d). All these experiments were performed on 30 mM KCl-contracted rings. Statistical difference is indicated by (*a*) between treatment and control groups and by (*b*) between treatment and L-NAME groups (*$P<0.05$, **$P<0.01$). Results are mean ± s.e.m. of 6–8 experiments.

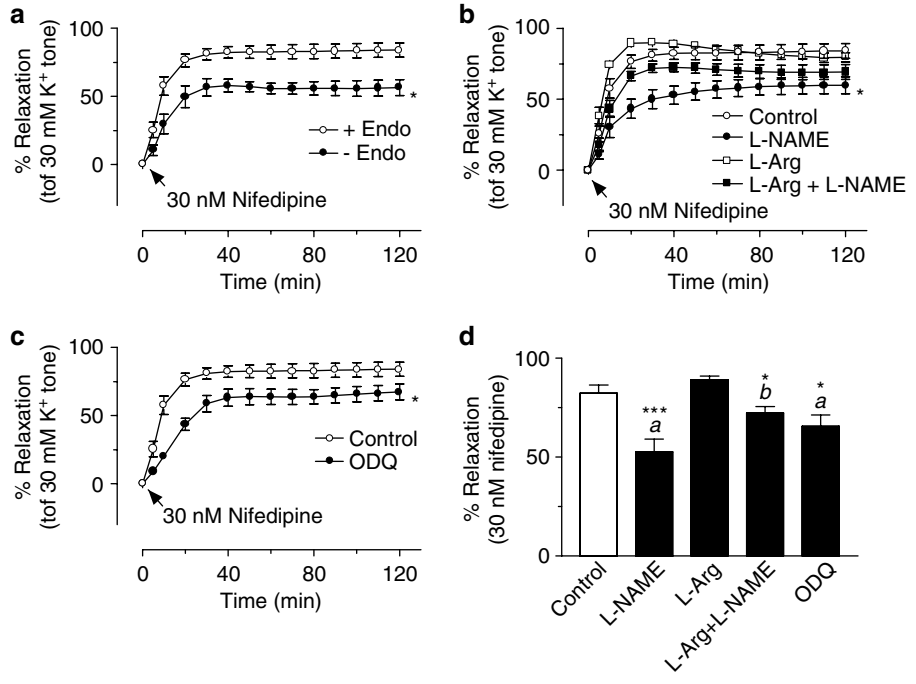

Figure 3 (a) Reduced relaxation to 30 nM nifedipine in rings without endothelium compared to rings with endothelium. Attenuated nifedipine-induced relaxation in the presence of L-NAME (b) or ODQ (c) in rings with endothelium. Treatment with L-arginine reversed the effect of L-NAME (b). The relaxant effect recorded at 30 min after application of nifedipine (d). All these experiments were performed on 30 mM KCl-contracted rings. Statistical difference is indicated by *a* between treatment and control groups and by *b* between treatment and L-NAME groups (*$P<0.05$, ***$P<0.001$). Results are mean ± s.e.m. of 6–8 experiments.

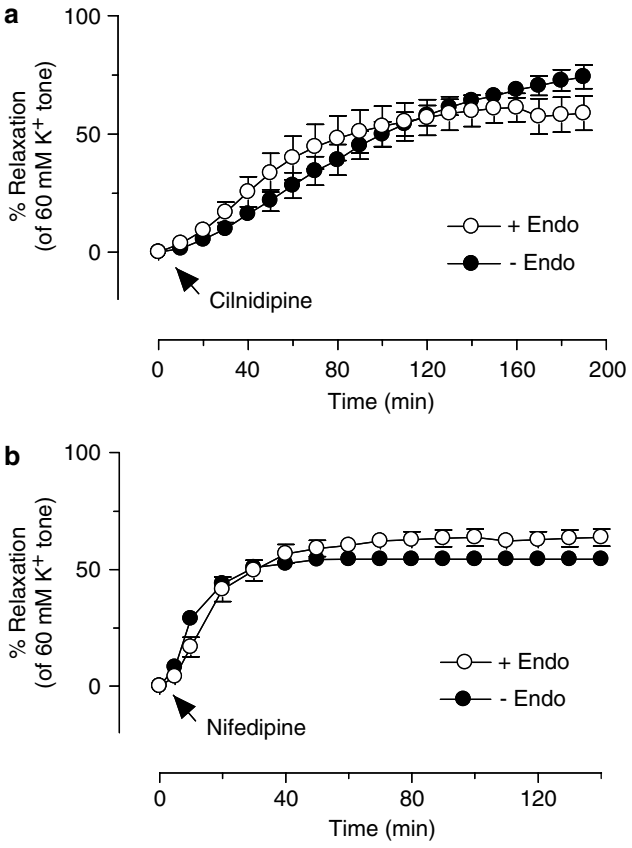

Figure 4 Lack of influence of the endothelium on relaxation to 30 nM cilnidipine (a) or 30 nM nifedipine (b) in 60 mM KCl-contracted rings. Results are mean ± s.e.m. of five experiments.

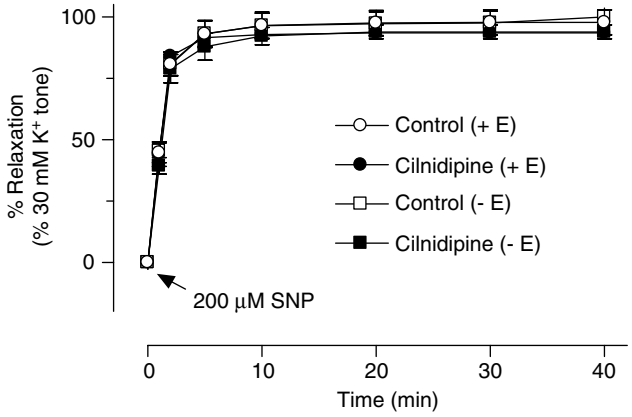

Figure 5 Lack of the effect of cilnidipine (10 nM, 30-min incubation) on relaxation to 200 μM sodium nitroprusside in 30 mM KCl-contracted rings with and without endothelium. Results are mean ± s.e.m. of five experiments.

Free radical scavenging effect

As compared with a known antioxidant, vitamin E, cilnidipine, and nifedipine at concentrations (1–100 nM) that relaxed porcine coronary arteries did not possess significant free radical-scavenging activity (Figure 7).

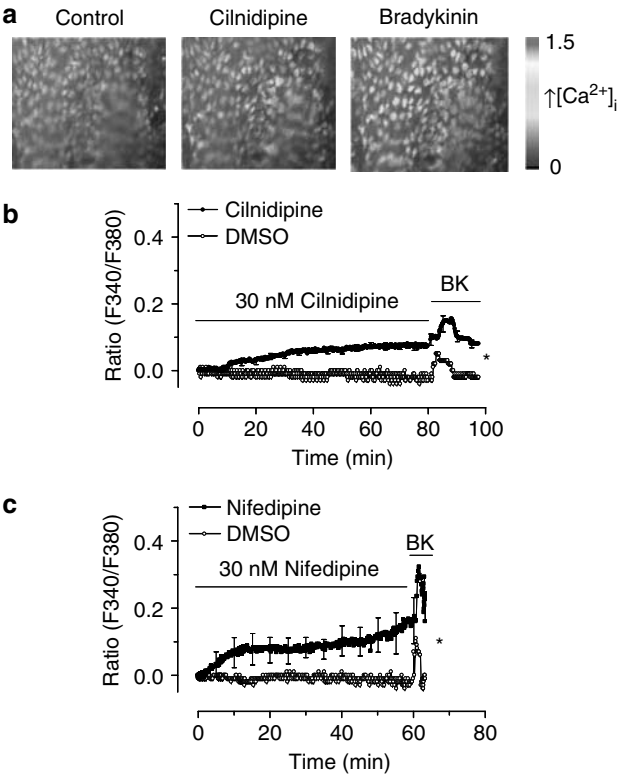

Figure 6 The stimulatory effects of cilnidipine (30 nM, a, b) and nifedipine (30 nM, c) on endothelial calcium levels *in situ* in cut-open rings with endothelium. The addition of bradykinin further increased the endothelial $[Ca^{2+}]_i$. Statistical difference between two curves is indicated by $^*P<0.05$. Results are mean ± s.e.m. of 4–5 experiments from different pigs.

Discussion

It is known that some DHPs can augment NO release from endothelial cells either in cultured cells or noncoronary arteries (Zhang & Hintze, 1998; Berkels *et al*., 2001) probably through the upregulation of the eNOS activity (Ding & Vaziri, 2000) or antioxidation (Berkels *et al*., 2001). The main findings of the present study include he following: (i) porcine coronary artery relaxations to cilnidipine and nifedipine are partly endothelium-dependent and involve NO/cyclic GMP signaling. (ii) The onset of cilnidipine's action is much slower as compared to that of nifedipine, but both DHPs showed similar relaxing potency. (iii) Like nifedipine, cilnidipine raises $[Ca^{2+}]_i$ in native endothelial cells in intact coronary arteries *in situ*, with similar onset as to that of cilnidipine-induced relaxation. (iv) Neither cilnidipine nor nifedipine possesses a free radical-scavenging effect.

NO, one of the most prominent endothelium-derived regulators of arterial tone, relaxes vascular smooth muscle cells *via* cyclic GMP-mediated signaling (Murad, 1994). Several DHPs including nifedipine (short-acting, first generation) and lacidipine (long-acting, second generation) increase the release of NO in endothelial cells, which is sensitive to inhibition by NOS inhibitors (Ding & Vaziri, 2000; Berkels *et al*., 2001; Krenek *et al*., 2001). The present results demonstrate that like nifedipine, cilnidipine (30 nM)-induced relaxation is partly endothelium-dependent and the L-NAME (an NOS inhibitor)- or ODQ (a guanylate cyclase inhibitor)-

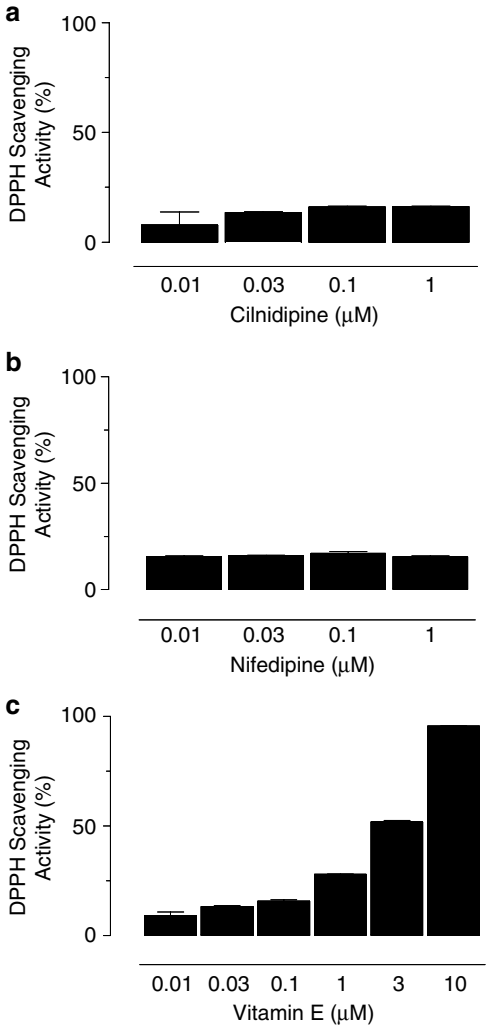

Figure 7 The free radical scavenging activity of DHPs, cilnidipine (a), nifedipine (b), and vitamin E (c). Results are mean ± s.e.m. of six experiments.

sensitive component accounts for approximately 30% of the total relaxation. Treatment of arteries with L-arginine prevented L-NAME-induced inhibition of relaxation. Furthermore, L-NAME did not alter the relaxant effects of cilnidipine or nifedipine in rings without endothelium. The results suggest that the endothelium-dependent relaxation to cilnidipine is mediated by NO/cyclic GMP-dependent mechanisms in porcine coronary arteries. The lack of an effect on relaxation induced by sodium nitroprusside indicates that cilnidipine does not interact with NO/cyclic GMP-mediated signaling pathway in vascular smooth muscle. The endothelium-dependent relaxation to either cilnidipine or bradykinin was observed only in 30 mM KCl- but not in 60 mM KCl-contracted rings, suggesting that the presence of 60 mM extracellular KCl abolishes endothelium-dependent relaxation in porcine coronary arteries. This may explain why a significantly less relaxation to cilnidipine was obtained in rings contracted by 60 mM KCl (61.6 ± 4.5% relaxation) than in rings contacted by 30 mM KCl (88.9 ± 4.5% relaxation). High KCl-induced contractions were comparable in rings with and without endothelium, indicating that the endothelium is unlikely to contribute tonically to DHP-induced relaxation. The present results indicate that the reported N-type Ca^{2+} channel-blocking activity in sympathetic nerves does not influence cilnidipine-induced relaxation since ω-conotoxin (an potent inhibitor of N-type Ca^{2+} channel blocker on sympathetic nerves) (Brock & Cunnane, 1999) was without effect.

The biosynthesis of NO by eNOS from L-arginine is a Ca^{2+}-dependent process. Various endothelial NO-dependent vasodilators stimulate the release of NO by increasing endothelial $[Ca^{2+}]_i$. Like nifedipine, cilnidipine acutely increased $[Ca^{2+}]_i$ in native endothelial cells of coronary arteries and this stimulatory effect relied on the presence of extracellular Ca^{2+} ions. A long-lasting time course of increases in $[Ca_i^{2+1}]$ (up to 80 min, Figure 6b) correlate well with the slowly developing relaxation to cilnidipine (Figure 2a). Likewise, 20 min is required for the steady response to nifedipine (30 nM) in both vessel tension and $[Ca^{2+}]_i$ (Figures 3a and 6c). A stimulatory effect of cilnidipine on $[Ca^{2+}]_i$ is unlikely to be related to L/N-type Ca^{2+} channels since these channels are not expressed in endothelial cells (Adams *et al*., 1989).

The acute endothelium-dependent effect of cilnidipine or nifedipine may not involve the upregulation of expression of eNOS mRNA and/or protein, since nifedipine starts to stimulate the release of NO 20 min after its application and prolonged treatment (48 h) did not affect eNOS protein levels (Berkels *et al*., 2001). However, long-term treatment (5–6 weeks) with lacidipine (Krenek *et al*., 2001) or cilnidipine (Kobayashi *et al*., 2001) was reported to elevate the eNOS mRNA levels in hypertensive rats. In addition, chronic nifedipine therapy (6 months) restored coronary endothelial function in patients with coronary artery disease (ENCORE Investigators, 2003). The present data together with others suggest that the mechanisms underlying the acute and chronic effects of DHPs on endothelial NO function may differ. The former is more likely coupled to an elevated endothelial $[Ca^{2+}]_i$ (Berkels *et al*., 1999), while the latter is associated with chronically increased eNOS expression and/or activity (Kobayashi *et al*., 2001; Krenek *et al*., 2001). However, we cannot discount the possibility that DHP Ca^{2+} antagonists induce vasorelaxation also by inhibiting the production and/or effects of endothelial vasoconstrictors.

Finally, some but not all DHP Ca^{2+} antagonists were reported to have an antioxidant activity (Lesnik *et al*., 1997; Berkels *et al*., 2001; Cominacini *et al*., 2003). Nifedipine (48 h treatment) increased the bioavailability of endothelial NO through reducing the production of reactive oxygen species (ROS) (Berkels *et al*., 2001), while shorter treatment (6 h) with nifeidpine or nimodipine did not affect the ROS formation (Cominacini *et al*., 2003). Although we cannot exclude a possible acute inhibition of the ROS production by cilnidipine in intact porcine coronary arteries, the present results show that cilnidipine or nifedipine displayed little or no free radical-scavenging effect. Therefore, the increased bioavailability of NO by removal of free radicals may not contribute to the endothelium-dependent effect of cilnidipine.

In summary, this study has provided evidence for one additional mechanism by which cilnidipine exerts its action on porcine coronary arteries, for example, stimulation of Ca^{2+}-dependent NO release in endothelial cells, apart from its blockade of Ca^{2+} channels in arterial smooth muscle and sympathetic nerves as demonstrated by other investigators. The increased bioavailability of NO may exert additional

antithrombotic, antiproliferative, and antiatherosclerotic effects. The N-type Ca^{2+} channel-blocking property of cilnidipine may offer clinical benefits by attenuating the reflex tachycardia due to sympathetic stimulation, a potentially fatal side effect resulting from nifedipine therapy in patients with coronary artery disease (Furberg *et al*., 1995; Stason *et al*., 1999) and by retaining Ca^{2+} channel-independent vascular benefits of nifedipine as shown in the present study. Indeed, recent clinical studies show that cilnidipine is effective as a once-daily antihypertensive drug without reflex tachycardia and increases in the sympathetic nerve activity in essential hypertensive patients (Minami *et al*., 2000; Kitahara *et al*., 2004). It has yet to be determined whether cilnidipine's stimulation of endothelial NO release is clinically relevant, for example, in the treatment of coronary artery disease and other chronic vascular diseases. Further studies are needed on the effects of chronic cilnidipine treatment on coronary endothelial dysfunction in hypertension.

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References

ADAMS, D.J., BARAKEH, J., LASKEY, R. & VAN BREEMEN, C. (1989). Ion channels and intracellular calcium in vascular endothelial cells. *FASEB J.,* **3,** 2389–2400.

BERKELS, R., EGINK, G., MARSEN, T.A., BARTELS, H., ROESEN, R. & KLAUS, W. (2001). Nifedipine increases endothelial nitric oxide bioavailability by antioxidative mechanisms. *Hypertension,* **37,** 240–245.

BERKELS, R., MUELLER, A., ROESEN, R. & KLAUS, W. (1999). Nifedipine and Bay K 8644 induce an increase of $[Ca^{2+}]_i$ and NO in endothelial cells. *J. Cardiovasc. Pharmacol. Ther.,* **4,** 175–181.

BLOIS, M.S. (1958). Antioxidant determination by the use of a stable free radical. *Nature,* **181,** 1199–1200.

BROCK, J.A. & CUNNANE, T.C. (1999). Effects of Ca^{2+} concentration and Ca^{2+} channel blockers on noradrenaline release and purinergic neuroeffector transmission in rat tail artery. *Br. J. Pharmacol.,* **126,** 11–18.

COMINACINI, L., FRATTA PASINI, A., GARBIN, U., PASTORINO, A.M., DAVOLI, A., NAVA, C., CAMPAGNOLA, M., ROSSATO, P. & LO CASCIO, V. (2003). Antioxidant activity of different dihydropyridines. *Biochem. Biophys. Res. Commun.,* **302,** 679–684.

DING, Y. & VAZIRI, N.D. (2000). Nifedipine and diltiazem but not verapamil up-regulate endothelial nitric-oxide synthase expression. *J. Pharmacol. Exp. Ther.,* **292,** 606–609.

ENCORE INVESTIGATORS. (2003). Effect of nifedipine and cerivastatin on coronary endothelial function in patients with coronary artery disease: the ENCORE I Study (Evaluation of Nifedipine and Cerivastatin On Recovery of coronary Endothelial function). *Circulation,* **107,** 422–428.

FUJII, S., KAMEYAMA, K., HOSONO, M., HAYASHI, Y. & KITAMURA, K. (1997). Effect of cilnidipine, a novel dihydropyridine Ca^{++}-channel antagonist, on N-type Ca^{++} channel in rat dorsal root ganglion neurons. *J. Pharmacol. Exp. Ther.,* **280,** 1184–1191.

FURBERG, C.D., PSATY, B.M. & MEYER, J.V. (1995). Nifedipine. Dose-related increase in mortality in patients with coronary heart disease. *Circulation,* **92,** 1326–1331.

HEMPEL, A., LINDSCHAU, C., MAASCH, C., MAHN, M., BYCHKOV, R., NOLL, T., LUFT, F.C. & HALLER, H. (1999). Calcium antagonists ameliorate ischemia-induced endothelial cell permeability by inhibiting protein kinase C. *Circulation,* **99,** 2523–2529.

HOSONO, M., FUJII, S., HIRUMA, T., WATANABE, K., HAYASHI, Y., OHNISHI, H., TAKATA, Y. & KATO, H. (1995). Inhibitory effect of cilnidipine on vascular sympathetic neurotransmission and subsequent vasoconstriction in spontaneously hypertensive rats. *Jpn. J. Pharmacol.,* **69,** 127–134.

HUANG, T.Y., CHU, T.F., CHEN, H.I. & JEN, C.J. (2000). Heterogeneity of $[Ca^{2+}]_i$ signaling in intact rat aortic endothelium. *FASEB J.,* **14,** 797–804.

JULIUS, S. (1993). Sympathetic hyperactivity and coronary risk in hypertension. *Hypertension,* **21,** 886–893.

KITAHARA, Y., SAITO, F., AKAO, M., FUJITA, H., TAKAHASHI, A., TAGUCHI, H., HINO, T., OTSUKA, Y., KUSHIRO, T. & KANMATSUSE, K. (2004). Effect of morning and bedtime dosing with cilnidipine on blood pressure, heart rate, and sympathetic nervous activity in essential hypertensive patients. *J. Cardiovasc. Pharmacol.,* **43,** 68–73.

KRENEK, P., SALOMONE, S., KYSELOVIC, J., WIBO, M., MOREL, N. & GODFRAIND, T. (2001). Lacidipine prevents endothelial dysfunction in salt-loaded stroke-prone hypertensive rats. *Hypertension,* **37,** 1124–1128.

KOBAYASHI, N., MORI, Y., MITA, S., NAKANO, S., KOBAYASHI, T., TSUBOKOU, Y. & MATSUOKA, H. (2001). Effects of cilnidipine on nitric oxide and endothelin-1 expression and extracellular signal-regulated kinase in hypertensive rats. *Eur. J. Pharmacol.,* **422,** 149–157.

LESNIK, P., DACHET, C., PETIT, L., MOREAU, M., GRIGLIO, S., BRUDI, P. & CHAPMAN, M.J. (1997). Impact of a combination of a calcium antagonist and a β-blocker on cell- and copper-mediated oxidation of LDL and on the accumulation and efflux of cholesterol in human macrophages and murine J774 cells. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 979–988.

LOHN, M., MUZZULINI, U., ESSIN, K., TSANG, S.Y., KIRSCH, T., LITTERAL, J., WALDRON, P., CONRAD, H., KLUGBAUER, N., HOFMANN, F., HALLER, H., LUFT, F.C., HUANG, Y. & GOLLASCH, M. (2002). Cilnidipine is a novel slow-acting blocker of vascular L-type calcium channels that does not target protein kinase C. *J. Hypertens.,* **20,** 885–893.

MINAMI, J., KAWANO, Y., MAKINO, Y., MATSUOKA, H. & TAKISHITA, S. (2000). Effects of cilnidipine, a novel dihydropyridine calcium antagonist, on autonomic function, ambulatory blood pressure and heart rate in patients with essential hypertension. *Br. J. Clin. Pharmacol.,* **50,** 615–620.

MINAMI, J., ISHIMITSU, T., KAWANO, Y., NUMABE, A. & MATSUOKA, H. (1998). Comparison of 24-h blood pressure, heart rate, and autonomic nerve activity in hypertensive patients treated with cilnidipine or nifedipine retard. *J. Cardiovasc. Pharmacol.,* **32,** 331–336.

MOMBOULI, J.V. & VANHOUTTE, P.M. (1999). Endothelial dysfunction: from physiology to therapy. *J. Mol. Cell Cardiol.,* **31,** 61–74.

MURAD, F. (1994). The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. *Recent Prog. Horm. Res.,* **49,** 239–248.

NAKAJIMA, M., YAMADA, S., UCHIDA, S. & KIMURA, R. (2002). *In vivo* measurement of 1,4-dihydropyridine receptors in mesenteric arteries of spontaneously hypertensive rats and effect of nifedipine and cilnidipine. *Biol. Pharm. Bull.,* **25,** 24–28.

PSATY, B.M., HECKBERT, S.R., KOEPSELL, T.D., SISCOVICK, D.S., RAGHUNATHAN, T.E., WEISS, N.S., ROSENDAAL, F.R., LEMAITRE, R.N., SMITH, N.L., WAHL, P.W., WAGNER, E.H. & FURBERG, C.D. (1995). The risk of myocardial infarction associated with antihypertensive drug therapies. *JAMA,* **274,** 620–625.

STASON, W.B., SCHMID, C.H., NIEDZWIECKI, D., WHITING, G.W., CAUBET, J.F., CORY, D., LUO, D., ROSS, S.D. & CHALMERS, T.C. (1999). Safety of nifedipine in angina pectoris: a meta-analysis. *Hypertension,* **33,** 24–31.

TADDEI, S., VIRDIS, A., GHIADONI, L., ULERI, S., MAGAGNA, A. & SALVETTI, A. (1997). Lacidipine restores endothelium-dependent vasodilation in essential hypertensive patients. *Hypertension,* **30,** 1606–1612.

TAKEDA, K., YAMAGISHI, R., MASUMIYA, H., TANAKA, H. & SHIGENOBU, K. (2004). Effect of cilnidipine on L- and T-type calcium currents in guinea pig ventricle and action potential in rabbit sinoatrial node. *J. Pharmacol. Sci.,* **95,** 398–401.

TOMINAGA, M., OHYA, Y., TSUKASHIMA, A., KOBAYASHI, K., TAKATA, Y., KOGA, T., YAMASHITA, Y., FUJISHIMA, Y., ABE, I. & FUJISHIMA, M. (1997). Ambulatory blood pressure monitoring in patients with essential hypertension treated with a new calcium antagonist, cilnidipine. *Cardiovasc. Drugs Ther.,* **11,** 43–48.

VARAGIC, J., SUSIC, D. & FROHLICH, E.D. (2002). Cilnidipine improves spontaneously hypertensive rat coronary hemodynamics without altering cardiovascular mass and collagen. *J. Hypertens.,* **20,** 317–322.

YOSHIMOTO, R., DOHMOTO, H., YAMADA, K. & GOTO, A. (1991). Prolonged inhibition of vascular contraction and calcium influx by the novel 1,4-dihydropyridine calcium antagonist cinaldipine (FRC-8653). *Jpn. J. Pharmacol.,* **56,** 225–229.

ZHANG, X. & HINTZE, T.H. (1998). Amlodipine releases nitric oxide from canine coronary microvessels: an unexpected mechanism of action of a calcium channel-blocking agent. *Circulation,* **97,** 576–580.

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