The Vasorelaxing Action of Labedipinedilol-A Involves Endothelial Cell–Derived NO and eNOS Expression Caused by Calcium Influx

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Abstract: Labedipinedilol-A, a novel dihydropyridine-type calcium antagonist, has been shown to induce hypotension and vasorelaxation. The objective of the present study was to investigate the effect of labedipinedilol-A on vascular function of rat aortic rings and cultured human umbilical vein endothelial cells (HUVECs). Labedipinedilol-A induced vasorelaxation in rat aortic rings that had been precontracted with phenylephrine in a concentration-dependent manner. This labedipinedilol-A-induced relaxation was significantly reduced by endothelium removal and by exposure to L-N^G-nitroarginine methyl ester (L-NAME), methylene blue, or 1*H*-[1,2,4]oxadiazolol[4,3,*a*]quinoxalin-1-one (ODQ). In addition, the cyclic GMP content was significantly increased by labedipinedilol-A, which was inhibited by L-NAME in aorta. In cultured HUVECs, labedipinedilol-A induced concentration-dependent formation of NO and Ca^{2+} influx, and it increased the abundance of endothelial NO synthase (eNOS) protein. Furthermore, labedipinedilol-A suppressed basal, 10% FBS- and thrombin-stimulated endothelin-1 production, which were reversed by pretreatment with L-NAME, demonstrating that NO was able to inhibit production of ET-1 in HUVECs. Labedipinedilol-A significantly protected cultured HUVECs against dihydroxyfumarate/iron ion-induced decrease of glutathione and cell death. Moreover, labedipinedilol-A also inhibited iron-induced lipid peroxidation in rat brain homogenate and scavenged 2,2′-azobis (2-amidinopropane) dihydrochloride-derived peroxy radicals. Labedipinedilol-A acts as lacidipine with additional antioxidant effects and can protect endothelial cells against free radical-induced lipid peroxidation and cell injury. Our results indicate that the endothelium-dependent vasorelaxation by labedipinedilol-A is mediated through Ca^{2+}-dependent activation of NO synthase and stimulation of NO/cyclic GMP pathway.

Key Words: labedipinedilol-A, vasorelaxation, endothelium, nitric oxide, cyclic GMP, Ca^{2+}

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Calcium antagonists are clinically useful vasodilators, used widely in the treatment of hypertension and ischemic heart disease. They lower blood pressure by a well-characterized mechanism of blocking L-type calcium channels in smooth muscle cells.[1] Additionally, there is growing evidence that dihydropyridine-type calcium antagonists also modulate endothelial function by other mechanisms[2] because macrovascular endothelial cells do not express L-type calcium channels. Labedipinedilol-A (Fig. 1) is a novel 1,4-dihydropyridine-type calcium antagonist synthesized in our laboratory.[3] As demonstrated earlier, labedipinedilol-A has been shown to lower blood pressure with less reflex tachycardia both in anesthetized normotensive rats and conscious spontaneously hypertensive rats. Labedipinedilol-A has been suggested to possess calcium entry and α/β-adrenoceptor blocking activities in a single molecule, which is different from conventional calcium antagonists such as nifedipine and almodipine.[4] Labedipinedilol-A not only is a vasodilator but also inhibits translocation of ventricle PKCε and various forms of humoral signaling, including endothelin-1, in DOCA-salt-treated rats.[5] In addition, labedipinedilol-A is an inhibitor of vascular smooth muscle cell proliferation induced by a broad group of mitogens, such as serum, platelet-derived growth factor, and norepinephrine, which may have great potential in the prevention of progressive atherosclerosis.[6]

A number of studies have demonstrated that different calcium antagonists, such as isradipine, nisoldipine, and lacidipine, enhance endothelial NO release in a variety of models and in different species.[2,7] Because endothelial cells do not express L-type calcium channels, the increased NO release must be caused by another non-L-type channel-mediated effect such as the antioxidative action.[8] Several calcium antagonists have been demonstrated to possess antioxidant and oxygen free radical–scavenging properties.[9,10] Superoxide anion can remove NO by combining chemically with it, forming the potent oxidant and cytotoxic radical peroxynitrite. Thus, calcium antagonists may potentially increase NO availability through removal of superoxide anion. NO released under physiological conditions is thought to have free radical–scavenging

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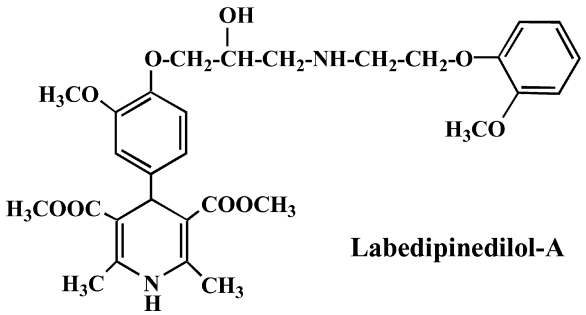

FIGURE 1. Chemical structure of labedipinedilol-A.

properties.[11] It has also been shown that NO can induce glutathione (GSH) synthesis in both endothelial and vascular smooth muscle cells.[12,13] This is a potentially important supplement to the antioxidant functions of NO associated with scavenging of oxygen radicals. Calcium antagonists have also been shown to prevent glutathione loss,[14] and this might also be a possible mechanism. In the past, some authors have taken cultured endothelial cells as a model system to assess cell injury from oxygen radical–mediated stress.[8,14]

The aim of the present study was to further investigate the mechanism underlying labedipinedilol-A-induced vasorelaxation. This question was addressed in thoracic aorta rings isolated from rats and in cell cultures of HUVECs. We found that labedipinedilol-A induced endothelium-dependent vasorelaxation. The removal of endothelium substantially reduced the effect of labedipinedilol-A. We also measured labedipinedilol-A-induced NO production as well as the expression of the constitutive endothelial NO synthase (eNOS) protein. Because some dihydropyridine-type calcium antagonists exert antioxidative properties,[14–17] we additionally investigated whether labedipinedilol-A may provide a endothelial cytoprotective effect against free radical–mediated acute losses of glutathione and survival. The results of this study provide the possible mechanism of vasorelaxant actions of labedipinedilol-A, including its ability to activate a NO–cyclic GMP cascade, Ca^{2+} influx in HUVECs, and to inhibit Ca^{2+} influx in vascular smooth muscle cells (VSMCs).

MATERIALS AND METHODS

Animals

Wistar rats were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. Animals for in vitro experiments were killed after anesthesia with pentobarbital or ether to achieve muscle relaxation and inhibition of sensory reflex. They were housed under conditions of constant temperature and controlled illumination (light on between 7:30 and 19:30). Food and water were available ad libitum. This study was approved by the Animal Care and Use Committee of the Kaohsiung Medical University.

Drugs and Chemicals

Labedipinedilol-A, synthesized in our laboratory, was dissolved in 10% propylene glycol, 10% absolute alcohol, and 2% 1 N HCl at 10 mM. Serial dilutions were made in distilled water. Phenylephrine, methylene blue, EGTA, thrombin, nicardipine HCl, and MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] were obtained from Sigma Chemical (St Louis, MO). 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-one (ODQ) and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Research Biochemical International (Natick, MA). F12 nutrient mixture, fetal bovine serum (FBS), penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO BRL Life Technologies (Grand Island, NY). Anti-eNOS antibodies were obtained from BD Transduction Laboratories (USA). All other reagents used were from E. Merck (Darmstadt, Germany).

Isolated Thoracic Preparation and Measurement of Tension

The aortic rings were prepared as described previously.[4] At the beginning of each experiment, aortic rings were stretched to a resting tension of 1 g and then contracted with phenylephrine (PE, 1 μM), and, when the contractions had reached a plateau, the endothelial integrity of the preparations (E^+) or absence of endothelium (E^-) was verified by adding acetylcholine (1 μM) to the superfusate. Only the aortic rings with a vasorelaxant response of >70% inhibition of the preconstructed level were considered E^+. The preparations were then washed and allowed to equilibrate with Krebs solution for 45 minutes before being contracted a second time with PE. When a stable vasoconstriction in response to PE (1 μM) was reached (considered 100%), labedipinedilol-A was applied directly to the bathing solution in cumulative concentrations (1 nM to 100 μM). To examine the possible mechanisms of vasorelaxant effects of labedipinedilol-A, the aortic rings were pretreated with the soluble guanylyl cyclase (sGC) inhibitor methylene blue (10 μM) or ODQ (1 μM), a NOS inhibitor L-NAME (100 μM), for 30 minutes before addition of labedipinedilol-A.

Measurement of Cyclic GMP

Isolated thoracic aortas of similar size were incubated for 60 minutes in an organ bath containing Krebs solution, which was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. The arteries were treated for 60 minutes with the control bathing solution and solution containing labedipinedilol-A (0.1–10 μM) and 100 μM IBMX. After the incubation, the levels of cyclic GMP were measured with a commercially available radioimmunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The protein content of the pellet dissolved in 1 mL of 0.5 N NaOH was determined by the method of Bradford,[18] using bovine serum albumin (BSA) as standard.

In Vitro Cell Culture

Human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection, Rockville, MD) were cultured in F12 nutrient mixture medium supplemented with 10% FBS, 1.6 mmol/L L-glutamine, 30 mg/mL endothelial cell growth supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 U/mL heparin. HUVECs of the third to fifth passage were used for all experiments.

Western Blot Analysis

Endothelial cells were processed, and eNOS protein abundance was determined by Western blot analysis using anti-eNOS antibody (BD Transduction Laboratories, San Diego, CA) in a manner that was similar to that described in our earlier studies.[19]

Determination of Nitrite/Nitrate (NO_2^-/NO_3^-)

NO production was assessed from NO_x recovered in the extracellular medium. NO_x was measured using an automated NO detector–high-performance liquid chromatography system (ENO-20, Kyoto, Japan) as previously described.[19] The amount of NO_x produced was normalized against total cellular protein, which was measured with a Bio-Rad kit (Bio-Rad Inc, Hercules, CA).

Ca^{2+} Measurements in HUVECs

Trypsinized cells (1×10^6 cells/mL) were loaded with 2 μM of the ester form of fura-2 (fura-2/acetoxy methyl) for 30 minutes at 25°C in DMEM. After loading, the cells were kept in a BSS (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 5, pH 7.4. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 mL of BSS and 5×10^5 cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Shimadzu, Japan) by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 2-second intervals. Maximum and minimum fluorescence values were obtained adding 10 μM digitonin (plus 10 mM $CaCl_2$) and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described assuming a K_d of 224 nM.[20]

Determination of Endothelin-1

HUVECs were grown in 24-well plates containing modified F12 medium supplemented as described above. Before the initiation of drug treatment, medium was aspirated, and the cells were washed once with fresh F12 medium. The cells then received 0.5 mL of F12 medium, supplemented without serum. Test agents were then added to the cells at various concentrations and allowed to incubate for 30 minutes at 37°C. After drug treatment, FBS (10%) or thrombin (10 U/mL) was added to the cells, which were then incubated for an additional 24 hours. At the end of the experiment, the medium was collected and centrifuged at $16,500 \times g$ for 5 minutes to remove cellular debris. Viable cells were solubilized in 0.5 N NaOH, and protein was determined according to the method of Bradford using BSA as the standard. Endothelin-1 (ET-1) concentrations were measured using a commercial ELISA kit (Phoenix Pharmaceuticals, Inc, Belmont, CA).

Measurement of Antioxidative Effects

Trypsinized endothelial cells were seeded at a density of 1×10^4 cells/cm^2 in Corning 24-well culture plates in F12 medium. After 24 hours of cell growth, the medium was removed and replaced with 500 μL of the balanced salt buffer containing 10 mM glucose, 125 mM NaCl, 1.2 mM $MgCl_2$, and 10 mM KH_2PO_4, pH 7.2. To assess the drug effects, labedipinedilol-A, nifedipine, amlodipine, nicardipine, or trolox was preincubated with the cells for 60 minutes in the balanced salt buffer before exposure (except for the control) to the iron-catalyzed free radical system consisting of 0.83 mM dihydroxyfumarate and 0.025 mM $FeCl_3$ chelated 0.25 mM ADP. The incubations are continued up to 60 minutes, and samples are assayed for glutathione loss and cell survival.

Endothelial Glutathione Loss and Cell Survival

Total cellular glutathione [unoxidized glutathione (GSH) + ½ oxidized glutathione (GSSG)] was assayed by the "recycling method," which combines the calorimetric reaction of 5,5′-dithiobis-2-nitrobenzoic acid with the enzymatic specificity of glutathione reductase as provided by a commercially available assay (Cayman Chemical, Ann Arbor, MI). Oxidized glutathione was measured by the same assay except for prior masking of GSH by 1% (vol/vol) 2-vinyl pyridine. Cellular survival/viability 24 hours after the oxidant exposure was determined by the tetrazolium/MTT (Sigma) assay in 24-well plates. Briefly, MTT, dissolved in PBS at 2 mg/mL, was added to all wells (125 μL/500 μL medium) and then incubated for an additional 4 hours at 37°C. Finally, 800 μL of acidified isopropanol (0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The supernatants were retrieved, briefly centrifuged, and read within 30 minutes using a test wavelength of 570 nm and a reference of 700 nm.

Lipid Peroxidation and Peroxy Radical Scavenging Activities

The rates of membrane lipid peroxidation were measured by the formation of thiobarbituric acid-reactive substance (TBARS). Rat brain homogenates (1 mL) were incubated at 37°C for 5 minutes with 10 μL of labedipinedilol-A, nifedipine, amlodipine, nicardipine, trolox or vehicle. Lipid peroxidation was initiated by the addition of 0.1 mL of 0.25 mM $FeCl_2$ and 1 mM ascorbic acid.[21] After 30 minutes of incubation, the reaction was stopped by adding 0.1 mL of 0.2% butylated hydroxytoluene. Thiobarbituric acid was then added, and the mixture was heated for 30 minutes in a boiling water bath. TBARS was extracted by *n*-butanol and measured at 532 nm. The amount of TBARS was quantified using the linear regression obtained from malondialdehyde standards.

The scavenging abilities of labedipinedilol-A and trolox on aqueous peroxy radicals were determined by the method described by Tsuchiya et al.[22] The reaction mixture (2 mL) consists of 0.5 nM B-phycoerythrin, 25 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) in 75 mM sodium phosphate buffer (pH 7.4). The stoichiometric factors of the test compounds with hydrophilic peroxy radicals were calculated according to the lag time during which fluorescence loss was protected (excitation at 540 nm and emission at 570 nm). Ascorbic acid was used as a positive control.

Statistics

The results are expressed as mean ± SEM, and they were analyzed by Student *t*-test or 1-way analysis of variance (ANOVA). When the ANOVA manifested a statistical difference, the Bonferroni *t* or Student-Newman-Keuls test was

applied. A *P* value less than 0.05 was considered to be significant in all experiments. Analysis of the data and plotting of the figures were carried out with the aid of computer software (SigmaStat and SigmaPlot, Version 5.0, San Rafael, CA) run on an IBM-compatible computer (IBM, White Plains, NY).

RESULTS

Effects of Labedipinedilol-A on NO Synthase and Soluble Guanylyl Cyclase

As shown in Figure 2, cumulative concentrations of labedipinedilol-A (1 nM to 100 μM) produced concentration-dependent relaxations both in endothelium-denuded (E^-) and endothelium-intact (E^+) aortic rings. Labedipinedilol-A showed a significant shift in the concentration–response curve after endothelium denudation, suggesting that at least a part of the observed effect is endothelium-dependent. The relaxations of endothelium-intact aortic rings elicited by labedipinedilol-A (ED_{50} 0.15 μM) were significantly inhibited by pretreatment with 100 μM L-NAME (ED_{50} 0.88 μM), 1 μM ODQ (ED_{50} 0.58 μM), or 10 μM methylene blue (ED_{50} 1.57 μM), respectively (Fig. 2).

Effects of Labedipinedilol-A on Cyclic GMP Accumulation in Rat Aortic Segments

The basal cyclic GMP level was 2.12 ± 0.21 pmol/mg protein. Labedipinedilol-A (1, 10 μM) significantly increased the cyclic GMP levels in a concentration-dependent manner. Treatment with 10 μM labedipinedilol-A for 60 minutes significantly increased it to 2-fold (4.12 ± 0.25 pmol/mg protein). However, the rise of cyclic GMP was fully eliminated by pretreatment with L-NAME (100 μM) (Fig. 3).

Effects of Labedipinedilol-A on eNOS Protein Expression

A time-dependent increase in eNOS protein expression was found with 10 μM labedipinedilol-A (Fig. 4A). Compared with the control basal band, the expression of eNOS protein was also increased by treatment with labedipinedilol-A concentration-dependently (Fig. 4B), and the level was significantly increased to 131.16 ± 5.34% at 1 μM and 182.35 ± 4.22% at 10 μM (Fig. 4C).

Effects of Labedipinedilol-A on NO_x Production in HUVECs

The average basal NO_x content in cultured HUVECs was 65.69 ± 1.72 pmol/μg proteins. Labedipinedilol-A (1, 10 μM) significantly stimulated NO_x production in a concentration-dependent manner in HUVECs (Fig. 5). The vehicle itself did not significantly alter the basal NO_x in HUVECs. Preincubation with L-NAME (100 μM) resulted in an almost complete inhibition of the NO_x formation of the cells (Fig. 5).

Effects of Labedipinedilol-A on $[Ca^{2+}]_i$ in HUVECs

The average basal $[Ca^{2+}]_i$ in HUVECs was 83.06 ± 9.07 nM (n = 12). Cell treatment with 0.1 μM labedipinedilol-A did not significantly alter the basal $[Ca^{2+}]_i$ (92.39 ± 2.38 nM). Labedipinedilol-A (1 and 10 μM) induced a $[Ca^{2+}]_i$ elevation in HUVECs in normal-Ca^{2+} BSS in a concentration-dependent

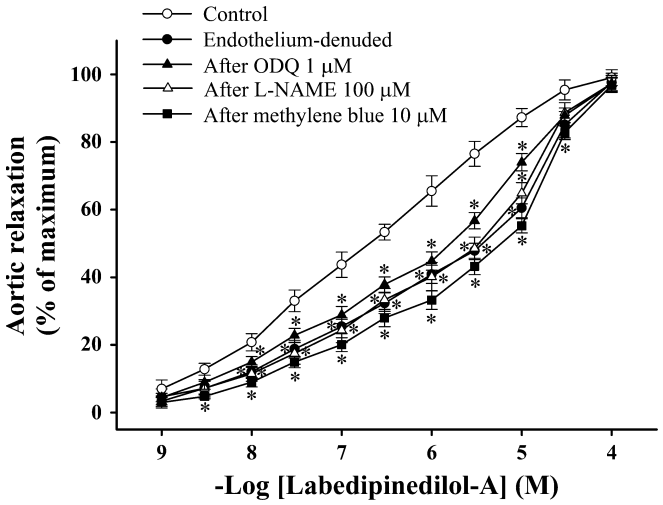

FIGURE 2. Effects of endothelium-denuded L-NAME (100 μM), methylene blue (10 μM), and ODQ (1 μM) on labedipinedilol-A–induced relaxation in rat aorta preconstricted with phenylephrine (1 μM). Each point represents the mean ± SEM of 8 experiments. *$P < 0.05$ versus endothelium-intact rings. Two-way repeated-measures ANOVA followed by Student-Newman-Keuls test. Phenylephrine was added to the bath to induce a stable contraction of the aortic strips; the response was taken as 100%.

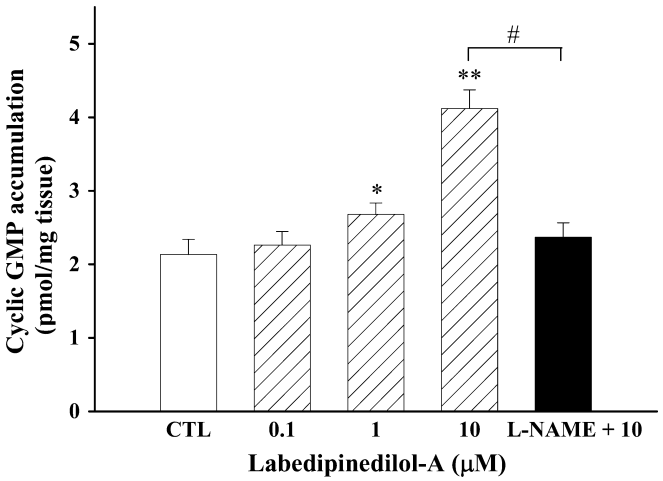

FIGURE 3. Effects of labedipinedilol-A on cyclic GMP accumulation in the tissue segments of rat aorta. Isolated tissue segments of rat aorta were incubated for 60 minutes with the control bathing solution containing various concentrations of labedipinedilol-A in the presence of 100 μM IBMX. Each value represents the mean ± SEM of 3 independent triplicate experiments. *$P < 0.05$, **$P < 0.01$ versus control value, ANOVA followed by Bonferroni *t* test. #$P < 0.01$ versus stimulation with labedipinedilol-A (10 μM) alone, by use of paired Student *t* test. CTL, vehicle control.

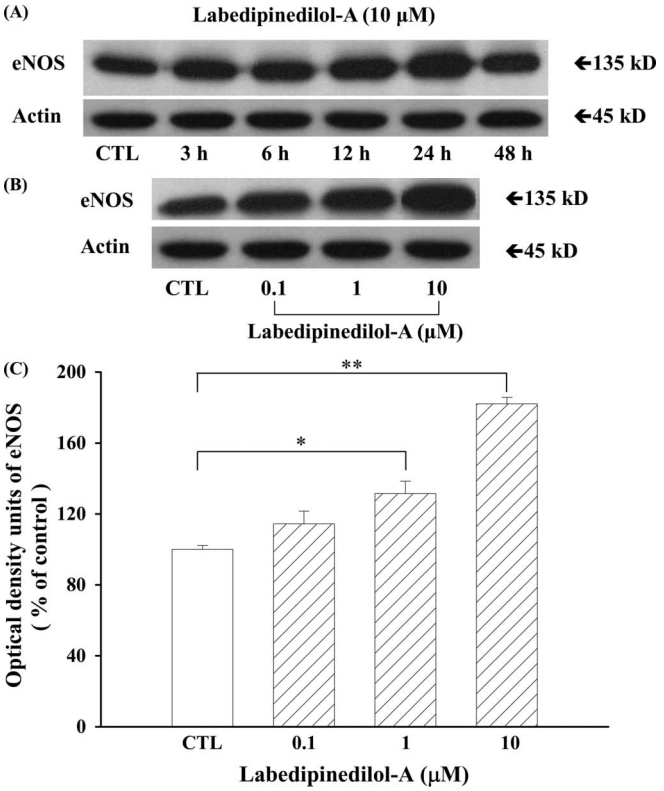

FIGURE 4. Effects of labedipinedilol-A on eNOS expression in HUVECs. A, HUVECs were cultured for indicated times with serum-free medium containing 10 μM labedipinedilol-A. B, Concentration-dependent effect of labedipinedilol-A on the expression of eNOS in HUVECs. C, Densitometry analyses are presented as the relative ratio of eNOS protein/actin protein. Each value represents the means ± SEM of 3 independent triplicate experiments. $*P < 0.05$, $**P < 0.01$ versus control value, ANOVA followed by Bonferroni *t* test. CTL, vehicle control.

manner (Fig. 6A). The peaks of $[Ca^{2+}]_i$ were 110.14 ± 7.19 nM and 125.65 ± 10.34 nM, respectively. Figure 6B shows that in Ca^{2+}-free medium, 1 μM thapsigargin induced a $[Ca^{2+}]_i$ transient with a maximum net value of 88.83 ± 8.52 nM. Conversely, there was no effect on $[Ca^{2+}]_i$ when cells were treated with 10 μM labedipinedilol-A in Ca^{2+}-free plus EGTA BSS. Thapsigargin, as a positive control, can induce an increase in Ca^{2+} influx, which has been attributed to a capacitative Ca^{2+} entry triggered by the depletion of an intracellular store.[23]

Effects of Labedipinedilol-A on ET-1 Production from HUVECs

As shown in Figure 7A, labedipinedilol-A suppressed basal (serum-free medium), 10% FBS- and thrombin-stimulated ET-1 production from HUVECs. The IC_{50} for labedipinedilol-A to inhibit ET-1 production in HUVECs were 4.52 μM, 6.54 μM, and 10.5 μM, respectively. In the presence of 100 μM L-NAME, labedipinedilol-A (10 μM) was not able to reduce ET-1 production (Fig. 7B).

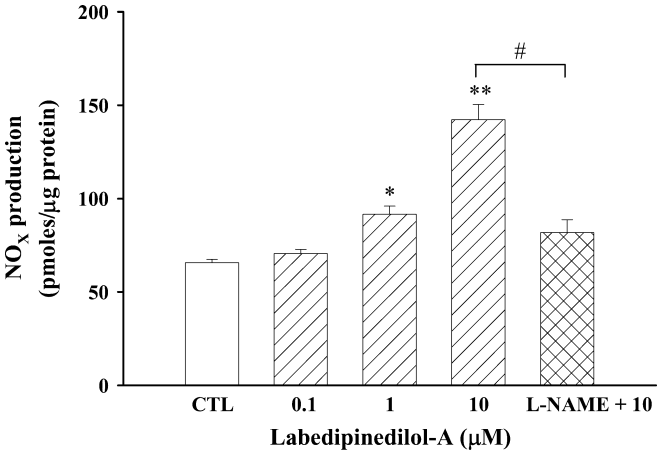

FIGURE 5. The nitric oxide (NO_x) production measured by the level of NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), in HUVECs. Data are shown for HUVECs (1×10^6 cells/well) with or without preincubation for 24 hours with labedipinedilol-A. Each value represents the mean ± SEM of 4 independent triplicate experiments. $*P < 0.05$, $**P < 0.01$ versus control value, ANOVA followed by Bonferroni *t* test. $^{\#}P < 0.01$ versus stimulation with labedipinedilol-A (10 μM) alone, by use of paired Student *t* test. CTL, vehicle control.

Endothelial Protective Effects of Labedipinedilol-A Against Loss of Glutathione Mediated by Oxy-Radicals

As presented in Figure 8, the sample exposed to the oxy-radical generation system in the absence of the drug resulted in a 54% loss of total glutathione in 60 minutes. When the cells were pretreated with labedipinedilol-A (0.1–10 μM) before the addition of the free radical system, varying degrees of attenuation of the induced loss of glutathione were achieved. For comparison, trolox and 3 calcium antagonists (nifedipine, amlodipine, and nicardipine) were also selected to assess the glutathione loss. Pretreatment of the cells with each agent concentration-dependently protected against the loss of glutathione (Fig. 8) at 60 minutes of incubation. The order of potency of cytoprotective effects is trolox > nicardipine > labedipinedilol-A = amlodipine > nifedipine.

Protective Effects of Labedipinedilol-A on Cell Survival

As shown in Figure 9, when the cells were incubated with oxy radical alone, there was a 45% loss of cell viability compared with the controls. The data indicated that pretreatment of the attached endothelial cells for 60 minutes with each agent at a concentration of 0.1, 1, or 10 μM resulted in varying degrees of protection against cell death; the effects were, in general, concentration-dependent, and the order of efficacy appeared to be trolox > nicardipine > labedipinedilol-A = amlodipine > nifedipine. This order of efficacy was in good agreement with the degree of protection against acute loss of glutathione.

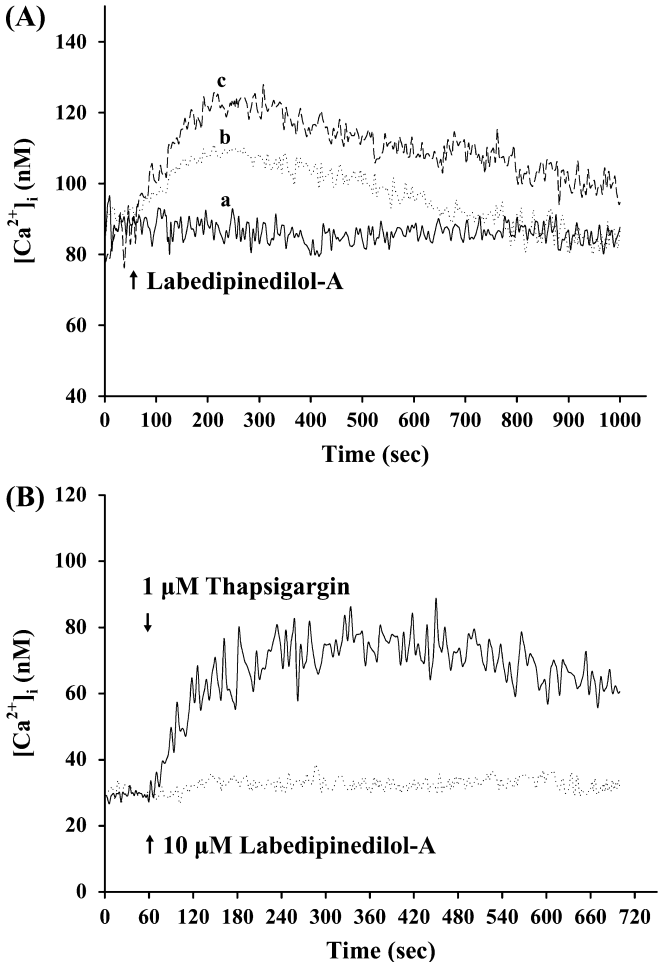

FIGURE 6. A, The concentration-dependent effect of labedipinedilol-A (0.1–10 μM) on free cytosolic Ca^{2+} concentration in the presence of extracellular Ca^{2+} in cultured HUVECs. The representative original recordings were obtained from 3 independent triplicate experiments. In Ca^{2+}-containing buffer, HUVECs were incubated with various concentrations of labedipinedilol-A (a, 0.1 μM; b, 1 μM; c, 10 μM). The changes in $[Ca^{2+}]_i$ were recorded for more than 15 minutes. B, In Ca^{2+}-free buffer, 1 μM thapsigargin or 10 μM labedipinedilol-A was added at 60 seconds. Then, the changes in $[Ca^{2+}]_i$ were recorded for more than 10 minutes.

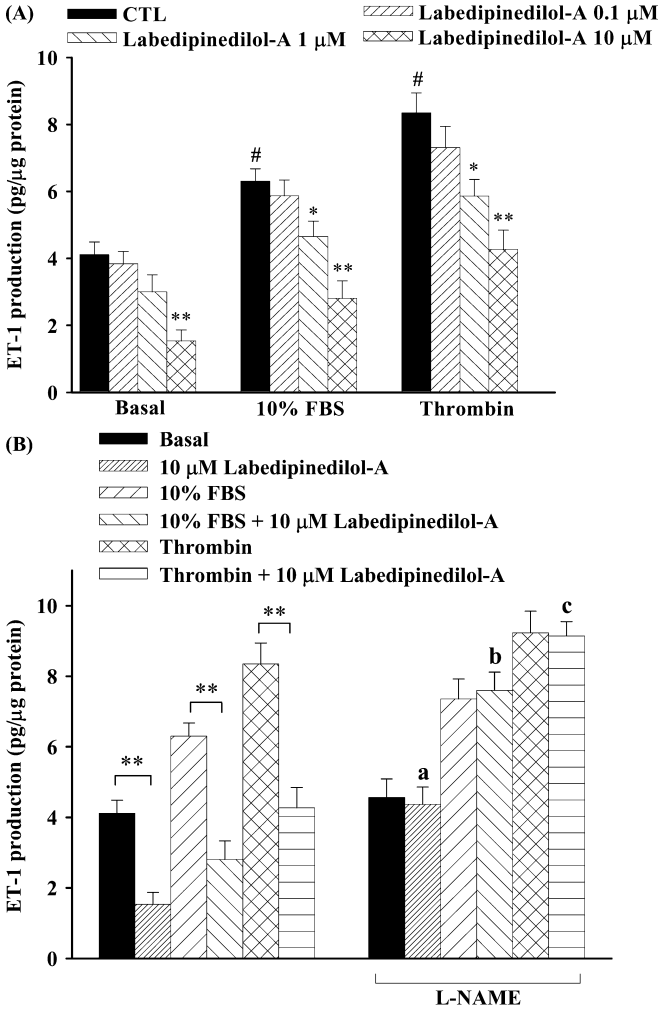

FIGURE 7. Effects of labedipinedilol-A on ET-1 production from HUVECs. A, Concentration-dependent effects of labedipinedilol-A on the basal, 10% FBS-, and thrombin-stimulated ET-1 production from HUVECs. Confluent HUVECs were incubated for 24 hours with serum-free medium containing various concentrations of labedipinedilol-A in the presence or absence of 10% FBS or 10 U/mL thrombin. B, Confluent HUVECs were incubated for 24 hours with serum-free medium containing 10 μM labedipinedilol-A, 10% FBS, or 10 U/mL thrombin in the presence or absence of 100 μM L-NAME. Each value represents the mean ± SEM of 3 independent triplicate experiments. $^{\#}P < 0.01$ versus CTL of basal; $^{*}P < 0.05$, $^{**}P < 0.01$ versus CTL value; a, $P < 0.01$ versus labedipinedilol-A (10 μM) alone; b, $P < 0.01$ versus 10% FBS + labedipinedilol-A; c, $P < 0.01$ versus thrombin + labedipinedilol-A, ANOVA followed by Bonferroni *t* test. CTL, vehicle control.

Inhibition of Lipid Peroxidation in Rat Brain Homogenates

The antiperoxidant activities of labedipinedilol-A and other test compounds were compared. Labedipinedilol-A, nifedipine, amlodipine, nicardipine, and trolox provided concentration-dependent inhibition of the lipid peroxidation; the IC_{50} values were determined to be 6.56 ± 0.24, 48.40 ± 0.96, 5.54 ± 0.59, 4.98 ± 0.18, and 4.07 ± 0.14 μM, respectively.

Peroxy Radical–Scavenging Activity in Aqueous Solution

When B-phycoerythrin was exposed to AAPH in sodium phosphate buffer, there was a loss of protein fluorescence intensity, the rate of which decreased on the addition of labedipinedilol-A (Fig. 10). The effect of labedipinedilol-A was concentration dependent. This fluorescence loss was also decreased by the addition of 10 μM trolox.

DISCUSSION

In the vascular system, NO is synthesized by eNOS in endothelial cells, and this stimulates cyclic GMP production

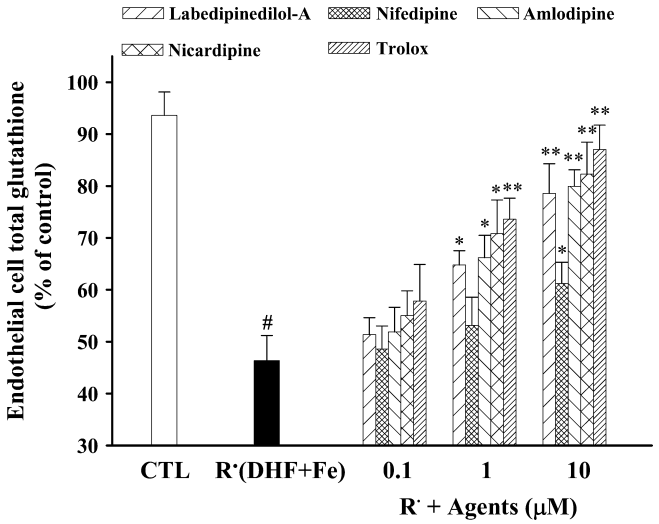

FIGURE 8. Effects of trolox and 4 calcium antagonists on dihydroxyfumarate [DHF + Fe-ADP]-mediated loss of endothelial glutathione. Confluent endothelial cells were preincubated with either calcium antagonists or trolox for 60 minutes before the addition of DHF + Fe-ADP. Each value represents the mean ± SEM of 3 independent triplicate experiments. $^{\#}P <$ 0.01 versus vehicle control, $^{*}P < 0.05$, $^{**}P < 0.01$ versus R• alone, ANOVA followed by Bonferroni *t* test. CTL, vehicle control.

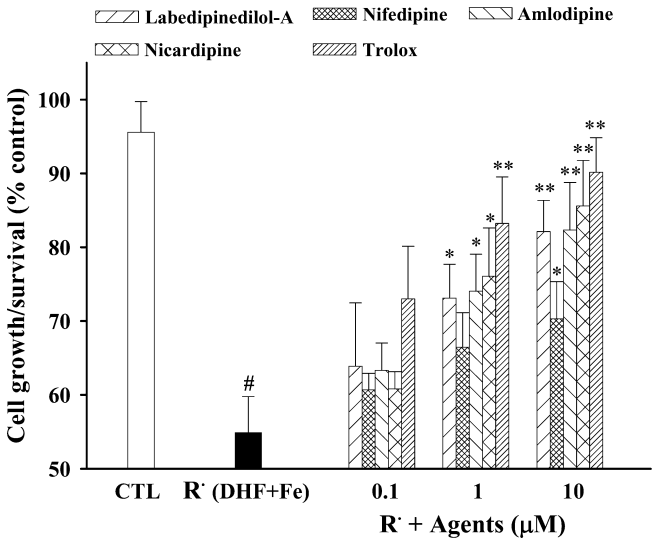

FIGURE 9. Protective effects of trolox and 4 calcium antagonists against free radical–mediated loss of cell survival. The attached cells, 24 hours after seeding at a density of $1 \times 10^4/cm^2$, were preincubated with calcium antagonists or trolox at different levels for 60 minutes before the incubation with free radical components R• (DHF + Fe-ADP) for 60 minutes; 24 hours later, cell proliferation was determined by the MTT absorbance assay. Each value represents the means ± SEM of three independent triplicate experiments. $^{\#}P <$ 0.01 versus vehicle control, $^{*}P < 0.05$, $^{**}P < 0.01$ versus R• alone, ANOVA followed by Bonferroni *t* test. CTL, vehicle control.

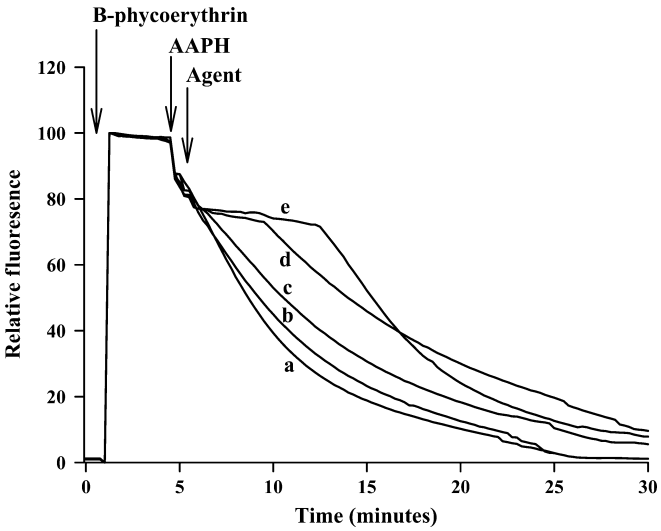

FIGURE 10. Effects of labedipinedilol-A and trolox on hydrophilic peroxy radical–induced degradation of B-phycoerythrin and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). Fluorescence intensity of B-phycoerythrin was measured by excitation at 540 nm and emission at 570 nm. The agent indicated the moment vehicle (a), labedipinedilol-A (b, 0.1 μM; c, 1 μM; d, 10 μM), or Trolox (e, 10 μM) was added to the reaction mixture. Shown is one of the representative tracings (n = 6).

by activating sGC in the adjacent smooth muscle. Increased cyclic GMP causes contracted muscle to relax, possibly through lowering the intracellular Ca^{2+}, most likely by increasing Ca^{2+} efflux to the extracellular space and Ca^{2+} reuptake into intracellular stores.[24] Labedipinedilol-A has been reported to relax isolated aortic rings.[4] Our present data confirm that labedipinedilol-A can indeed relax precontracted aortic rings. These results suggest the hypotensive effect of labedipinedilol-A can be explained by the direct relaxing effect of labedipinedilol-A on the vasculature. As indicated in the present study, labedipinedilol-A also has an endothelium-derived vasorelaxing effect. However, our data showed clearly that, in addition to its effect on the endothelium, labedipinedilol-A also has a direct vasorelaxing effect on denuded aorta, indicating that labedipinedilol-A also has a direct relaxing effect on VSMCs. The fact that labedipinedilol-A can act on both the endothelium and VSMCs shows that labedipinedilol-A has some unusual properties as a dihydropyridine derivate. It then becomes crucial to determine the mechanism of action of labedipinedilol-A in these 2 target tissues.

In the present study, pretreatment with L-NAME, a NOS inhibitor, or ODQ, a selective inhibitor of sGC, inhibited the labedipinedilol-A–induced relaxation to an extent similar to that seen in the endothelium-denuded preparations. Methylene blue, another sGC inhibitor, also potently diminished labedipinedilol-A–induced relaxation. Consistent with this is the increased cyclic GMP content in the labedipinedilol-A–treated aorta, indicating that cyclic GMP in smooth muscle cells plays an important role in the NO donor-induced relaxation. In another series of experiments, labedipinedilol-A also potentiated cyclic GMP production, which was blocked

by the pretreated NOS inhibitor, L-NAME. Taken together, the endothelium-dependent vasorelaxation induced by labedipinedilol-A is probably exerted through a NO–cyclic GMP pathway.

Three NO synthase (NOS) isoforms have been identified to date.[25] eNOS has been shown to be Ca^{2+}/calmodulin-dependent and expressed constitutively, mainly in endothelial cells. eNOS is activated mainly by an increase of cytosolic $[Ca^{2+}]$.[26] In the present study, we found that labedipinedilol-A significantly increased the release of NO from HUVECs, which was inhibited by pretreatment with the NOS inhibitor L-NAME. As shown by Western blot analysis, labedipinedilol-A treatment augmented eNOS protein mass present in endothelial cells. Furthermore, Ca^{2+} influx, as shown by the increase of fura-2 fluorescence, was induced by labedipinedilol-A in HUVECs. These data indicate that labedipinedilol-A enhances the release of NO in blood vessels by an increase in the intracellular concentration of Ca^{2+} in endothelial cells initiating a cascade of reactions that lead to the activation of eNOS and enhanced release of NO.

Present studies revealed that Ca^{2+} is involved in the mechanism of action of labedipinedilol-A in both VSMCs and EC. From our previous report,[4] KCl-stimulated increased $[Ca^{2+}]_i$ was inhibited by labedipinedilol-A in VSMCs. KCl depolarizes voltage-dependent Ca^{2+} channels through which Ca^{2+} entry increases.[27] The fact that labedipinedilol-A reduces the increase in $[Ca^{2+}]_i$ elicited by KCl suggests that labedipinedilol-A may inhibit voltage-dependent Ca^{2+} channel activity, resulting in decreased Ca^{2+} entry and $[Ca^{2+}]_i$. In addition to Ca^{2+} channel inhibition, labedipinedilol-A also affects the release of intracellular store Ca^{2+}. Norepinephrine produced a transient contraction in isolated aorta in the absence of Ca^{2+} in bath. The contraction induced by norepinephrine was due to the release of stored Ca^{2+}.[28] Labedipinedilol-A was effective in inhibiting the NE-induced contraction in Ca^{2+}-free buffer. These data provide evidence that labedipinedilol-A inhibits both extracellular Ca^{2+} entry and release of intracellular store Ca^{2+} in VSMCs. The effects of labedipinedilol-A on endothelial function may probably involve the NO system. It also is known that the constitutive NO system in EC is stimulated by an increase in $[Ca^{2+}]_i$.[29] Then the question is whether the effect of labedipinedilol-A on EC is related to Ca^{2+}. If so, labedipinedilol-A has to increase $[Ca^{2+}]_i$. Indeed, in our fura-2 studies in HUVECs, labedipinedilol-A augmented $[Ca^{2+}]_i$ by increasing calcium influx. This is interesting because labedipinedilol-A has an opposite Ca^{2+}-regulating effect in VSMCs and its adjacent EC; both can produce vasorelaxation.

ET-1 production is inhibited by the release of endothelial NO.[30] A balance between these relaxing and contracting factors is important in the regulation of vascular tone. In our experiments, labedipinedilol-A enhanced the NO-induced suppression of ET-1 production from endothelial cells. However, labedipinedilol-A had no effect in the presence of the NO synthesis inhibitor L-NAME. It is suggested that reactive oxygen free radicals are the agent that caused the enhanced release of ET-1 from endothelial cells.[31] Moreover, inhibition of ET-1 production has been observed with the free radical scavenger superoxide dismutase, as this free radical scavenger inhibits ET-1 biosynthesis in cultured porcine aortic endothelial cells.[29] Thus, the inhibition of ET-1 production by labedipinedilol-A may contribute to its vasodilating and antioxidant actions.

NO is an important protective molecule via its vasodilator, antioxidant, antiplatelet, and antineutrophil actions. However, NO is detrimental if it combines with $O_2-^{\bullet}$ to form $ONOO^-$ which rapidly decomposes to highly reactive oxidant species leading to endothelial injury. $ONOO^-$ is detoxified if it combines with reduced glutathione to form S-nitrosoglutathione.[32] Glutathione is present in the cells mainly in reduced form, and the loss of glutathione results from its oxidative depletion by lipid peroxides.[17] It is believed that the cellular glutathione system is the major antioxidant defense system capable of detoxifying lipid peroxides and free radicals.[33] In the present study, we used 2 well-defined in vitro models to compare the differential antioxidant potency among labedipinedilol-A, trolox, and 4 calcium antagonists. With the membrane lipid peroxidation model, we demonstrated that the antiperoxidative potency of labedipinedilol-A is more than 7-fold more potent than nifedipine. With the cultured endothelial cell model, based on acute loss of glutathione mediated by iron-dependent $HO^{\bullet}$-generating system, it was estimated that labedipinedilol-A was more effective than nifedipine as an endothelial cytoprotectant (Fig. 9). Recently, different observations have suggested that dihydropyridine-type calcium antagonists increased endothelial NO bioavailability by an antioxidative protection.[8,9,17,34] As shown in Figures 8–10, labedipinedilol-A possesses both free radical-scavenging and lipid peroxidation inhibitory properties. Therefore, it seems that at least part of the increased NO release by labedipinedilol-A is due to a prevention of the NO inactivation by oxidative mechanisms.

In conclusion, the present study demonstrates that labedipinedilol-A did act both at the level of SMC and on endothelial cells. On one hand, it acted by blocking Ca^{2+} influx in SMC and on the other by promoting the influx of extracellular Ca^{2+} in endothelial cells. This latter action was likely responsible for release of NO from vascular endothelium. In addition to its vasorelaxant effect, labedipinedilol-A also exerts antioxidant effect. These results indicate that labedipinedilol-A increases endothelium-dependent vasorelaxation by restoring NO availability, an effect probably determined by antioxidant activity. This is in agreement with another dihydropyridine calcium antagonist, such as lacidipine, has been shown to inhibit oxidative stress, thereby reducing lipid peroxidation and protecting against free radical-induced injury in cultured endothelial cells.[2,7,15,35] Taken together, these findings indicate that labedipinedilol-A not only lowers blood pressure values but also has beneficial effects on vascular endothelium, thereby offering considerable potential in the prevention and/or treatment of atherosclerosis.

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