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Activation of NAD(P)H:quinone oxidoreductase ameliorates spontaneous hypertension in an animal model via modulation of eNOS activity

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Aims Hypertension is one of the most common human diseases worldwide, and extensive research efforts are focused upon the identification and utilizing of novel therapeutic drug targets. Nitric oxide (NO) produced by endothelial NO synthase (eNOS) is an important regulator of blood pressure (BP). β-Lapachone (βL), a well-known substrate of NAD(P)H:quinone oxidoreductase (NQO1), increases the cellular NAD^+/NADH ratio via the activation of NQO1. In this study, we evaluated whether βL-induced activation of NQO1 modulates BP in an animal model of hypertension.

Methods and results Spontaneously hypertensive rats (SHR), primary human aortic endothelial cells (HAEC), and endothelial cell lines were used to investigate the hypotensive effect of βL and its mode of action. βL treatment stimulated endothelium-dependent vascular relaxation in response to acetylcholine in aorta of SHR and dramatically lowered BP in SHR, but the hypotensive effect was completely blocked by eNOS inhibition with ω-nitro-L-arginine methyl ester. Aortic eNOS phosphorylation and eNOS protein expression were significantly increased in βL-treated SHR. *In vitro* studies revealed that βL treatment elevated the intracellular NAD^+/NADH ratio and concentration of free Ca^{2+} ($[Ca^{2+}]i$), and resulted in Akt/AMP-activated protein kinase/eNOS activation. These effects were abolished by NQO1 siRNA and $[Ca^{2+}]i$ inhibition through a ryanodine receptor blockade.

Conclusion This study is the first to demonstrate that NQO1 activation has a hypotensive effect mediated by eNOS activation via cellular NAD^+/NADH ratio modulation in an animal model. These results provide strong evidence suggesting NQO1 might be a new therapeutic target for hypertension.

Keywords eNOS • Hypertension • NAD^+/NADH ratio • NQO1 • β-Lapachone

1. Introduction

Uncontrolled hypertension is a key risk factor for cerebrovascular accidents, myocardial infarction, cardiac failure, arterial aneurysm, and chronic renal failure.[1] Although many anti-hypertensive medications have been developed and extensive research efforts focus on the exploitation of novel therapeutic drug targets, the percentage of hypertensive patients with blood pressures (BPs) in the control range (usually <140/90 mmHg) remains low.[1–4]

Nitric oxide (NO), an endothelium-derived relaxing factor,[5] is an important regulator of vascular tone and BP and is synthesized by endothelial NO synthase (eNOS).[6] Blocking eNOS with pharmacological inhibitors causes significant peripheral vasoconstriction and elevation of BP.[7] The major determinants of NO synthesis by eNOS include phosphorylation and intracellular concentration of free Ca^{2+} ($[Ca^{2+}]i$).[8–10] In endothelial cells, phosphorylation of the eNOS carboxy-terminus at serine^{1177} (Ser^{1177}) is crucial to eNOS activation.[11,12] Protein kinase B/Akt is the major kinase for eNOS phosphorylation at Ser^{1177},[11] and

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AMP-activated protein kinase (AMPK) also plays an important role in regulating eNOS protein expression.[13] In endothelial cells, store-operated Ca^{2+} entry regulates many endothelial functions.[14] eNOS, a Ca^{2+}/calmodulin-dependent enzyme, is activated by an increase in $[Ca^{2+}]i$.[15] Moreover, this $[Ca^{2+}]i$ has been known to be modulated by ryanodine receptors (RyRs) and the endoplasmic reticulum Ca^{2+} channels. RyRs activity is regulated by cyclic ADP-ribose (cADPR), a metabolite of NAD^+.[16] Previously, it was reported that treatment with an intracellular Ca^{2+} chelator inhibited eNOS-Ser^{1177} phosphorylation,[17] while stimulation of Ca^{2+} flux by an ionophore increased Ser^{1177} phosphorylation.[18]

NAD(P)H:quinone oxidoreductase (NQO1), a homodimeric enzyme initially identified in 1958, catalyses the oxidation of NADH to NAD^+ by various quinines,[19] and significantly reduced NAD^+/NADH ratios were observed in the liver and kidneys of $NQO1^{-/-}$ mice.[20] Several activators and substrates of NQO1 have been identified. One well-known NQO1 substrate is β-lapachone (βL; 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione).[21,22] βL extract, isolated from the bark of the Lapacho tree, has been used as a folk medicine for centuries[23] and initial studies have demonstrated its ability to inhibit tumour growth.[24]

Recent studies have indicated that βL increases the NAD^+/NADH ratio via NQO1 activation. Moreover, an increased cytoplasmic NAD^+/NADH ratio has beneficial effects on several features of metabolic syndrome, including obesity amelioration in mice and prevention of arterial smooth muscle cell proliferation in rats.[25,26] In this study, we observed that the increased NAD^+/NADH ratio resulting from NQO1 activation lowered BP via aortic endothelial eNOS modulation in spontaneously hypertensive rats (SHR). Here, we have introduced the experimental results and the underlying mechanism.

2. Methods

2.1 Materials

βL was chemically synthesized by Mazence Co (Suwon, Korea). ω-Nitro-L-arginine methyl ester (L-NAME), acetylcholine, phenylephrine, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (BABTA-AM), ethylene glycol tetraacetic acid (EGTA), 8-Br-cADPR, ES936, nicotinamide, LY249002, Wortmannin, compound C and 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) were purchased from Sigma Chemical Co (St Louis, MO, USA). Dicoumarol was purchased from Calbiochem (San Diego, CA, USA). Anti-eNOS antibody was purchased from BD Biosciences (San Diego, CA, USA). Anti-phospho-eNOS-Ser^{1177}, Anti-phospho-eNOS-Thr^{495}, anti-phospho-Akt-Ser^{473}, anti-phospho-Akt, anti-phospho-AMPK-Thr^{172}, anti-AMPK, anti-phospho-ACC-Ser^{79}, and anti-ACC antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-NQO1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Animals and experimental procedures

Twelve-week-old male SHR were purchased from an established colony at the Japan SLC (Inasa Production Facility, Japan). Experimental animals were assigned to three groups: (i) Group 1 included two subgroups that received either a regular chow diet or a diet containing βL (Mazence Co., Korea; 0.12%, *wt*/*wt*) for 3 days; (ii) Group 2 included two subgroups that received either a regular chow diet or a diet containing βL for 4 weeks; and (iii) Group 3 comprised three subgroups, a control group that received a regular chow diet for 6 weeks, a βL group that received βL in the diet for 6 weeks, and a βL + L-NAME group that received the same diet with βL group for the first 3 weeks followed by the addition of eNOS inhibitor L-NAME to drinking water for 7 days during Week 4 and 6 (50 mg/kg/day). All rats were acclimatized to a 12 h light/dark cycle at 22 ± 2°C for 2 weeks with unlimited food (standard chow diet; Research Diets Inc., New Brunswick, NJ, USA) and water in a pathogen-free facility. All animal experiments were approved by the Institutional Animal Use and Care Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3 Cell culture

Primary human aortic endothelial cells (HAEC) and mouse brain endothelial (bEnd.3) cells were purchased from ATCC. HAEC were cultured in endothelial basal medium-2 (EBM-2) medium supplemented with EGM-2 bullet kits (Endothelial Growth Medium-2; Lonza, Basel, Switzerland). bEnd.3 cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Hyclone Laboratory, UT, USA) supplemented with 10% foetal bovine serum, 0.5% bovine serum albumin, 250 μmol/L dimethyl sulfoxide, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere (5% CO_2/95% air) at 37°C.

2.4 Measurement of BP

Systolic BP (SBP) and diastolic BP (DBP) were measured in all animals each week following treatment. The rats were preheated to dilate their tail vessels first for 15–20 min at 39°C in a warming box and then BP was measured using the non-invasive tail cuff method in a restrainer heated electrically to 39°C. The animals were pre-trained with the apparatus (Softron BP-98A, Tokyo, Japan) at least three times before measurements, and BP was determined.

2.5 Measurement of cellular total NAD^+/NADH ratio

Cells were harvested with 80% methanol and lysed mechanically using a 31-gauge needle. Electrospray ionization mass spectrometry was performed in positive ion mode as described previously (see Supplementary materials online, *Methods*).[27]

2.6 Determination of pyruvate/lactate ratio

The pyruvate/lactate ratio representing the cytosolic free NAD^+/NADH ratio[28] was measured with a D-lactate assay kit (Megazyme International, Bray, Ireland) according to the manufacturer's instructions (see Supplementary materials online, *Methods*).

2.7 Organ-bath study for vascular reactivity

We used 12-week-old male SHR that had received either a regular chow diet or βL in the diet (0.12% *wt*/*wt*) for 4 weeks. The animals were anaesthetized (50 mg/kg sodium pentobarbital, ip), and the adequacy and the depth of anaesthesia condition was monitored based on paw-pinch test. After then, the thoracic aorta was immediately excised and immersed in an ice-cold modified Krebs solution. The aorta was cleansed of all adherent connective tissue using wet filter paper soaked in Krebs bicarbonate solution, and cut into four ring segments (4 mm in length) as described in a previous study.[29] The rings were not denuded of endothelium, and two stainless steel triangles were inserted through each vessel ring. Each aortic ring was suspended in a water-jacketed organ bath (22 mL), maintained at 37°C, and aerated with a mixture of 95% O_2/5% CO_2. One triangle was anchored to a stationary support, and the other was connected to an isometric force transducer (Grass FT03C; Grass Instruments, Quincy, MA, USA). The rings were stretched passively by imposing the optimal resting tension, 2.0 g, which was then maintained throughout

the experiment. Each ring was equilibrated in an organ-bath solution for 90 min. The viability and endothelial integrity of the aortic ring were confirmed with the contractile response to 10^{-6} mol/L phenylephrine and 10^{-7} mol/L acetylcholine. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP; AD Instruments Pty. Ltd., Castle Hill, Australia). For the measurement of contractile response, aortic rings were pre-treated with 10^{-6} mol/L phenylephrine and subjected to the cumulative addition of acetylcholine (10^{-9}–10^{-5} mol/L). Endothelium-dependent vasodilation was determined by generating dose–response curves for acetylcholine. Vasorelaxation in response to acetylcholine was expressed as a percentage contraction, determined by the percentage of inhibition to the pre-constriction tension. Endothelium-dependent NOS-independent vasorelaxation was assessed by generating dose–response curves to acetylcholine in rings pre-treated with the NOS inhibitor L-NAME (10^{-4} mol/L). Endothelium-independent vasodilation was measured by vasorelaxation in response to cumulative sodium nitroprusside in rings pre-constricted with phenylephrine (10^{-6} mol/L).

2.8 Measurement of eNOS activity

The conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline was used to assess eNOS activity in cultured cells using the NOS activity assay kit (Cayman Chemicals, Inc., MI, USA) according to the manufacturer's instructions (see Supplementary materials online, *Methods*).

2.9 Measurement of $[Ca^{2+}]i$

The $[Ca^{2+}]i$ in cells was measured with fluorescent dye fluo-4/AM (Invitrogen, Carlsbad, CA, USA) and confocal microscopy (LSM 510 META; Carl Zeiss, Jena, Germany) according to standard procedures (see Supplementary materials online, *Methods*).[30]

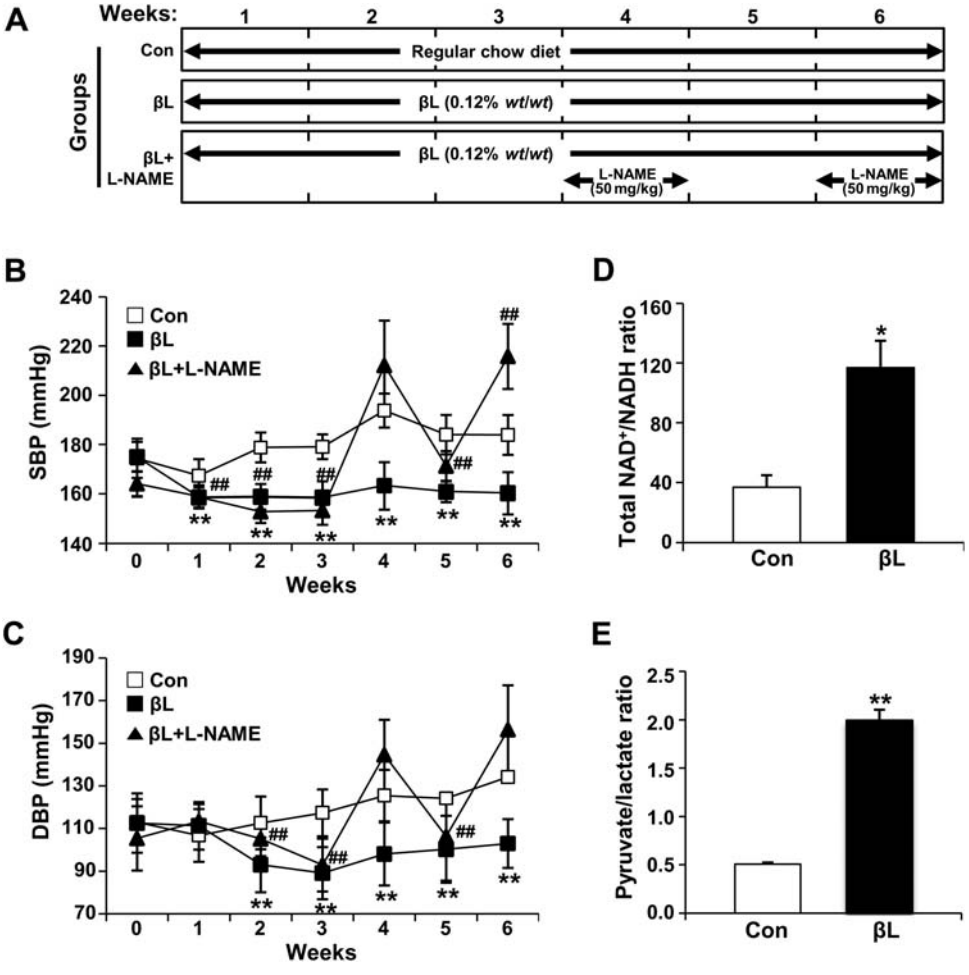

Figure 1 βL reduces BP in SHR and induces NADH oxidation in endothelial cells. (*A*) Experimental schedule of βL and/or L-NAME treatment. L-NAME was added to drinking water for 7 days during Week 4 and 6 (50 mg/kg/day) in the βL + L-NAME group. (*B* and *C*) BP was measured six times using the tail cuff method. SBP and DBP measurements were analysed on the basis of the resting SBP. The SBP (*B*) and DBP (*C*) were evaluated using the mean value of six measurements under stable-heart rate conditions. Control group, $n = 5$; βL group, $n = 6$; βL + L-NAME group, $n = 6$; grouped quantitative data are presented as means ± SEM. ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, βL group compared with control group; $^{\#\#}P < 0.01$, βL + L-NAME group compared with control group. The cellular NAD^+/NADH ratio was analysed in bEnd.3 cells. (*D*) Cells were treated with 10 μmol/L βL for 15 min. Treated cells were harvested with 80% methanol, and the supernatant was used for NAD^+ and NADH measurements. The NAD^+/NADH ratio was calculated from levels of total NAD^+ and total NADH measured by high performance liquid chromatography/mass spectrometry/mass spectrometry analysis. (*E*) Cells were treated with 10 μmol/L βL for 15 min. Treated cells were harvested with 1 mol/L perchloric acid. The supernatant was used for pyruvate and lactate measurements. Grouped quantitative data are presented as means ± SEM from a minimum of three independent experiments. Student's *t*-test, $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the control group.

2.10 Silencing of NQO1

At 30% confluence, bEnd.3 cells were transfected with HyperFect transfection reagent (Qiagen, Hilden, Germany) containing scrambled control siRNA (Invitrogen) or *Nqo1*-specific siRNA (AAACCAGCCUUU CAGAAUGGCUGGC; Invitrogen) following the manufacturer's instructions.

2.11 Adenoviral infection

Adenovirus encoding dnAMPKα was kindly provided by Dr In-Kyu Lee (Kyungpook National University School of Medicine),[26] and bEnd.3 cells were infected with adenoviral vectors.

2.12 Western blot analysis

Protein abundances and phosphorylation in isolated protein were analysed using western blot according to standard procedures (see Supplementary materials online, *Methods*).

2.13 Immunofluorescence staining

Paraffin sections were used for immunofluorescence staining. Following deparaffinizing and rehydration, the sections were treated with sodium citrate buffer (pH 6.5) and microwaved for 15 min. The sections were cooled for 30 min at room temperature and then incubated with 3% bovine serum albumin for 1 h. The tissue sections were incubated overnight at 4°C with anti-eNOS antibody (1:200). Alexa Fluoro 546 nm anti-mouse antibody (1:300) was then applied for 1 h at 37°C in the dark. Following each step, the samples were analysed under a dual fluorescence microscope (LSM 510 META; Carl Zeiss).

2.14 Statistical analyses

Numerical data are presented as means ± SEM. Comparisons between groups were performed using a two-tailed Student's *t*-test or one-way ANOVA test for multiple comparisons. The threshold of significance was set at $P < 0.05$.

3. Results

3.1 βL reduces BP in SHR and increases the NAD^+/NADH ratio in endothelial cells

As a result of βL supplementation in SHR, SBP and DBP were significantly reduced in both βL (159 ± 3 mmHg) and βL + L-NAME (154 ± 3 mmHg) groups (*Figure 1B* and *C*) when compared with the control (179 ± 3 mmHg) at 3 weeks post-treatment. However, following an additional week of L-NAME treatment, the BP-lowering effect of βL in the βL + L-NAME group was blocked, and the SBP rebounded to a level beyond that of the control group (212 ± 8 mmHg). The rebound-elevated BP in the βL + L-NAME group was reversed by the cessation of L-NAME administration for 1 week. These results were reproduced with a second treatment of L-NAME to the same group. In contrast, the lowered SBP level was maintained in the βL group (*Figure 1B* and *C*). These studies strongly support the model that βL decreases BP in a hypertensive animal model in an endothelium-dependent manner. Accordingly, we have used bEnd.3 cells to investigate whether βL increases the cellular NAD^+/NADH ratio via NQO1 activation in endothelial cells. The total cellular NAD^+/NADH ratio in mouse bEnd.3 cells was significantly increased by βL treatment (*Figure 1D*). The pyruvate/lactate ratio representing the cytosolic free NAD^+/NADH ratio[28] was also significantly increased by βL treatment (*Figure 1E*).

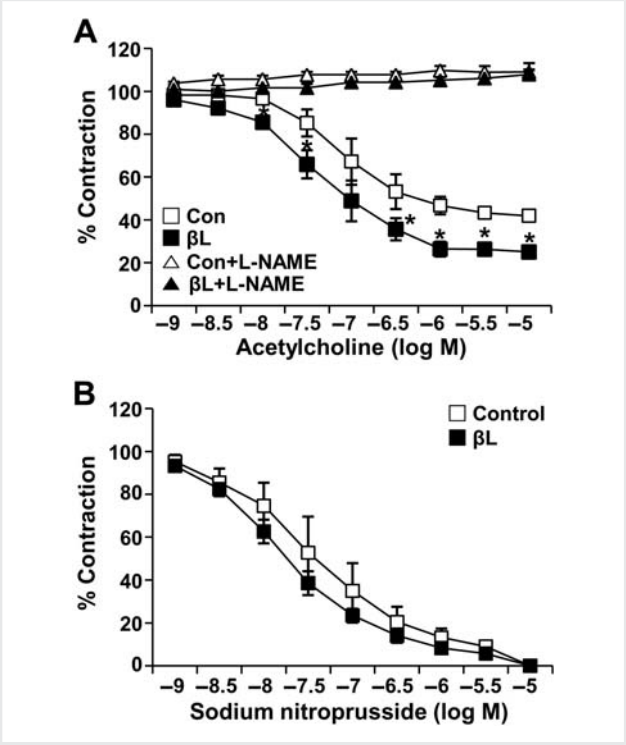

Figure 2 βL regulates endothelium-dependent vasodilation through eNOS. SHR received either a normal chow diet or a βL-supplemented diet (0.12% *wt/wt*) for 4 weeks. The thoracic aortas were extracted for the aortic ring test. (*A*) Endothelium-dependent vasodilation in response to acetylcholine (Ach), in the presence and absence of the NOS inhibitor L-NAME, was determined by measuring the relaxation of rings that had been pre-constricted with phenylephrine. (*B*) Endothelium-independent vasodilation was determined by measuring the relaxation of rings pre-constricted with phenylephrine in response to the NO donor sodium nitroprusside. $n = 3$ rings for each group; grouped quantitative data are presented as means ± SEM. Student's *t*-test, $^*P < 0.05$ compared with the control group.

3.2 βL improves vasomotor function in SHR

An *ex vivo* organ-bath study was performed to measure the effect of βL on aortic tension in SHR treated with βL for 4 weeks. Acetylcholine-induced (endothelium-dependent) vasorelaxation was improved in rings from βL-treated SHR (*Figure 2A*). In contrast, endothelium-independent vasodilation induced by the NO donor sodium nitroprusside was comparable between rings from control and βL-treated SHR (*Figure 2B*). Similarly, acetylcholine-induced vasodilation was absent in both control and βL-treated rings treated with L-NAME (*Figure 2A*). βL treatment, therefore, is believed to promote endothelium-dependent vasodilation and vascular bioavailability of NO through an eNOS-dependent mechanism.

3.3 βL stimulates eNOS activity through serine phosphorylation

Next, we used HAEC and bEnd.3 cells to investigate whether βL induces phosphorylation of eNOS at Ser^{1177}, a catalytically active phosphorylation site. As shown in *Figure 3A*, *B*, *C*, and *D*, βL stimulated eNOS-Ser^{1177} phosphorylation in a concentration- and time-

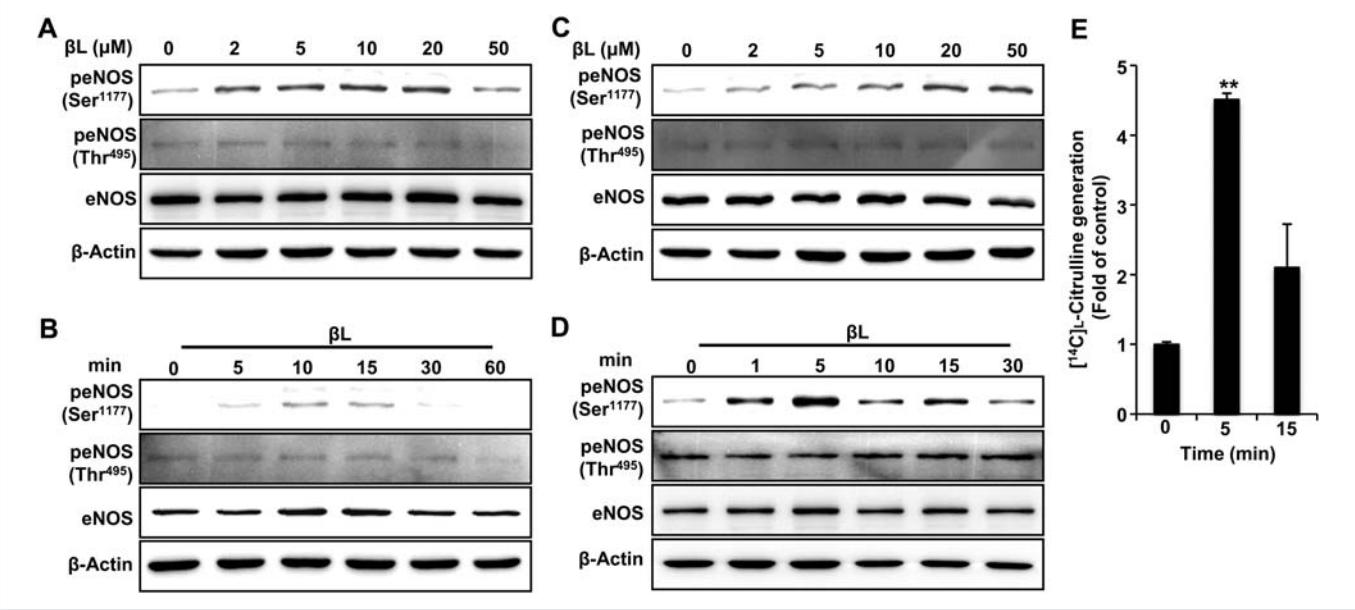

Figure 3 βL increases eNOS activity via Ser1177 phosphorylation. (*A* and *C*) HAEC (*A*) and bEnd.3 cells (*C*) were stimulated with the indicated concentrations of βL for (*A*) 15 or (C) 5 min, and eNOS phosphorylation was determined using immunoblot analysis using a phospho-specific antibody. (*B* and *D*) Representative western blots showing time-dependent changes in eNOS in HAEC (*B*) and bEnd.3 cells (*D*) stimulated with 20 μmol/L βL. (*E*) bEnd.3 cells were treated with 10 μmol/L of βL for 5 and 15 min, and eNOS activity was assayed by [^{14}C]L-citrulline production from [^{14}C]L-arginine. Results were normalized for total protein. Data for the control group are shown as 1 and average fold changes are displayed. Representative data from three independent experiments are shown.

dependent manner. However, eNOS-Thr^{495} phosphorylation (an inactive phosphorylation site) was not altered by βL treatment in both HAEC (*Figure 3A* and *B*) and bEnd.3 cells (*Figure 3C* and *D*). To investigate more directly whether βL can increase eNOS enzyme activity, the production of [^{14}C]L-citrulline was measured in bEnd.3 cells treated with βL. Within 5 min of βL treatment, [^{14}C]L-citrulline production was significantly increased (*Figure 3E*). On the basis of these results, we suggest that βL promotes eNOS activity by regulating Ser^{1177} phosphorylation.

3.4 βL-induced eNOS activation is dependent on PI3K/Akt and AMPK pathways

Ser^{1177} phosphorylation and subsequent eNOS activation in response to several distinct eNOS activators is mediated by PI3K/Akt or AMPK activation.[11–13] Therefore, we investigated whether βL activates Akt or AMPK, and whether the activation precedes eNOS-Ser^{1177} phosphorylation *in vitro*. Phosphorylation of both Akt and AMPK was detected within 5 min (HAEC) or 1 min (bEnd. 3 cells) of βL treatment. This is consistent with either, or both, signalling pathways being involved in eNOS activation (*Figure 4A* and *B*). To identify the kinase involved in eNOS-Ser^{1177} phosphorylation, bEnd.3 cells were treated with inhibitors of PI3K (LY249002) and AMPK (compound C) prior to βL treatment. *Figure 4C* shows that compound C had no effect on βL-induced eNOS phosphorylation. However, LY249002 dramatically inhibited the effect of βL on eNOS phosphorylation. These data indicate that βL-induced eNOS-Ser^{1177} phosphorylation is mediated through Akt signalling but not by AMPK.

To further explore our hypothesis that the AMPK pathway is not required for βL-induced eNOS phosphorylation, we used a dominant-negative AMPK (Ad-dnAMPKα) expressing adenovirus to inhibit AMPK in bEnd.3 cells. *Figure 4D* shows that AMPK activation by AICAR did not increase eNOS phosphorylation in bEnd.3 cells. Interestingly, in cells overexpressing Ad-dnAMPKα, βL seemed to diminish eNOS phosphorylation. This was due to decreased eNOS protein levels resulting from AMPKα inhibition, an observation consistent with a previous study.[13] Moreover, inhibition of endogenous AMPK by Ad-dnAMPKα transfection resulted in a time-dependent decrease in eNOS protein levels followed by a dnAMPKα increase (*Figure 4E*). These studies demonstrate that AMPK is also involved in βL-stimulated eNOS activation in endothelial cells through regulation of eNOS protein expression.

3.5 βL-stimulated phosphorylation of eNOS/Akt/AMPK is mediated by a NQO1-dependent increase in intracellular calcium

To investigate whether the βL-induced increase in the cellular NAD$^+$/NADH ratio (*Figure 1D* and *E*) can regulate [Ca^{2+}]i in endothelial cells, intracellular calcium fluxes were estimated using a Fluo-4/AM-based assay. βL led to increased [Ca^{2+}]i within 0.5 min. This [Ca^{2+}]i level was maintained until 1 min and then gradually decreased to basal levels by 2 min. The increased [Ca^{2+}]i resulting from βL treatment was completely abolished by blockade of [Ca^{2+}]i release with BABTA-AM. [Ca^{2+}]i release was partially suppressed by extracellular calcium chelation with EGTA (*Figure 5A*). The effect of βL on [Ca^{2+}]i was also suppressed using a RyR antagonist (8-Br-cADPR) and NQO1 inhibitors (dicoumarol and ES936) (*Figure 5A* and *B*). We next identified the molecular mechanisms underlying βL-induced intracellular calcium flux in endothelial cells.

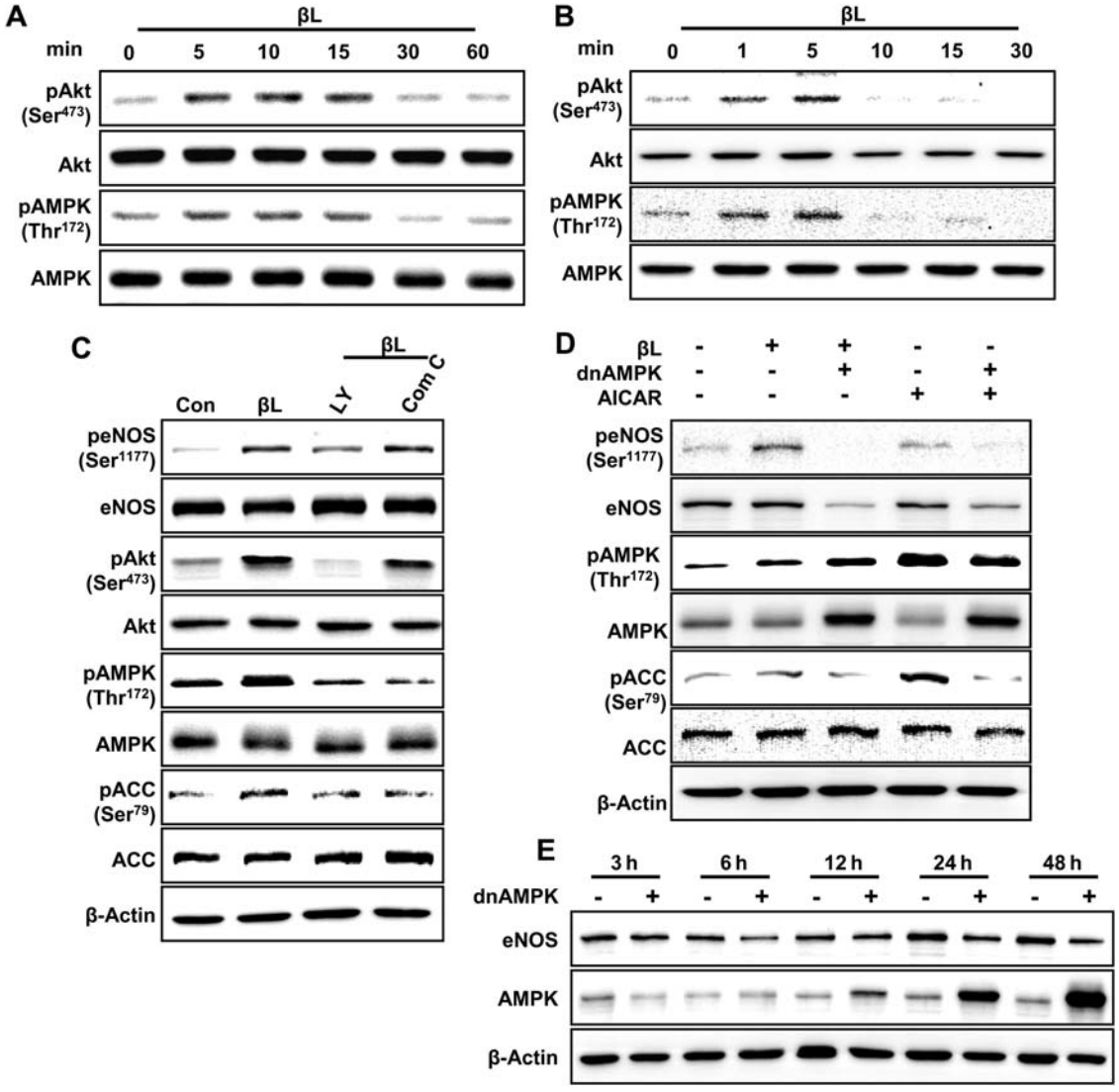

Figure 4 Phosphorylation of Akt and AMPK-mediated eNOS activation by βL. (*A* and *B*) HAEC (*A*) and bEnd.3 cells (*B*) were stimulated with 20 μmol/L of βL for the indicated time points, and active phosphorylation of Akt and AMPK was determined using immunoblot analysis using a phospho-specific antibody. (*C*) bEnd.3 cells were pre-treated with LY294002 (LY; 25 μmol/L) or compound C (Com C; 20 μmol/L) for 45 min, and treated with 20 μmol/L of βL for 5 min. Immunoblot analyses were performed using indicated antibodies. (*D*) bEnd.3 cells were infected with adenovirus encoding dnAMPKα for 60 h followed by stimulation with βL (20 μmol/L for 5 min) or AICAR (500 μmol/L for 1 h). (*E*) bEnd.3 cells were infected with adenovirus encoding dnAMPKα for the indicated times, and eNOS expression was determined by western blot. Representative blots from three independent experiments are shown.

Pre-treatment with BABTA-AM completely abolished βL-stimulated eNOS/Akt/AMPK phosphorylation. EGTA also partially inhibited phosphorylation of eNOS and Akt by βL (*Figure 5C*). RyR blockade with ADP-ribosyl cyclase inhibitor (nicotinamide) and 8-Br-cADPR also inhibited eNOS/Akt/AMPK phosphorylation by βL (*Figure 5D*). βL-induced phosphorylation of eNOS, Akt, and AMPK were all decreased by NQO1 inhibition, achieved by *Nqo1* siRNA transfection or ES936 treatment (*Figure 5E*). Intriguingly, pre-treatment with KN-93, a specific inhibitor of calcium/calmodulin-dependent protein kinase II (CaMKII), resulted in complete blockage of eNOS and Akt phosphorylation by βL (*Figure 5F*). These data strongly suggest that βL-induced increases in $[Ca^{2+}]i$ occur prior to eNOS activation resulting from Akt and AMPK phosphorylation in endothelial cells.

3.6 βL treatment increases aortic eNOS activity in SHR

We sought to confirm the aforementioned bEnd.3 *in vitro* data with an *in vivo* study of aortic eNOS activation in βL-treated SHR. eNOS and Akt phosphorylation increased after 3 days and 4 weeks of βL treatment. However, Akt phosphorylation then decreased within 6 weeks of βL treatment, corresponding with a reduction in eNOS phosphorylation (*Figure 6A*). Total eNOS protein levels were dramatically increased at all measured time points following βL treatment in SHR aorta. This was associated with increased AMPK phosphorylation (*Figure 6A*). The βL-induced increase in eNOS expression in the aorta was confirmed histologically. Intense

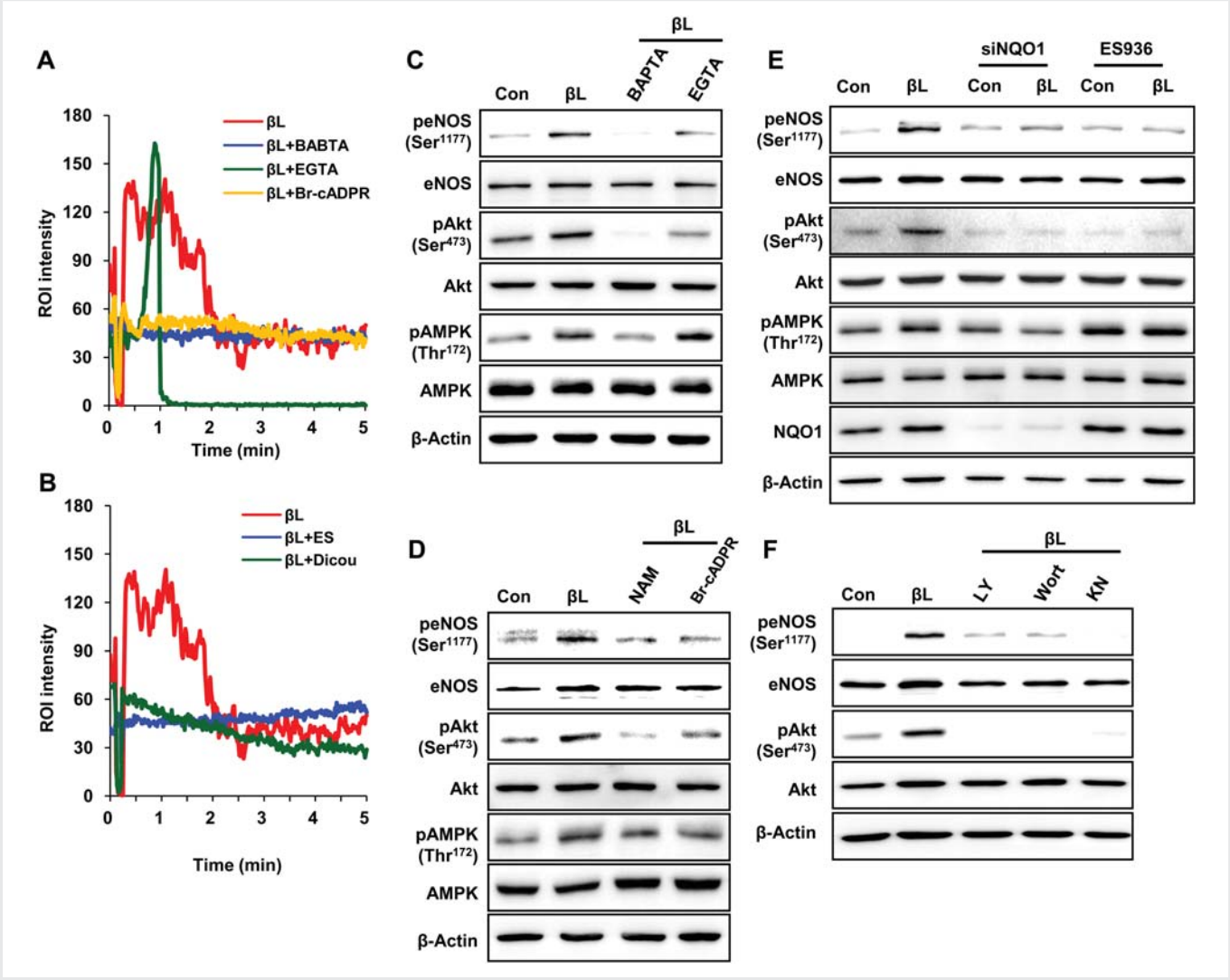

Figure 5 βL-induced increase of $[Ca^{2+}]i$ activates Akt/AMPK/eNOS via a NQO1-dependent mechanism. (*A* and *B*) $[Ca^{2+}]i$ in bEnd.3 cells was measured using the fluorescent dye fluo-4/AM and confocal microscopy. Cells were pre-treated with BABTA-AM (BABTA; 20 μmol/L), 8-Br-cADPR (Br-cADPR; 100 μmol/L), ES936 (ES; 5 μmol/L), or dicoumarol (Dicou; 5 μmol/L) for 45 min, or EGTA (2 mmol/L) for 2 min. During scanning, 20 μmol/L of βL was added and green fluorescence was detected each second for 5 min. Quantification of increases in relative fluorescence was measured using the regions of interest (ROI) tool. A representative graph from three independent experiments is shown. (*C* and *D*) bEnd.3 cells were pre-treated with BABTA (20 μmol/L), Br-cADPR (30 μmol/L), or nicotinamide (NAM; 10 mmol/L) for 45 min, or EGTA (2 mmol/L) for 2 min and stimulated with 20 μmol/L of βL for 5 min. (*E*) bEnd.3 cells were transfected with *Nqo1* siRNA for 48 h prior to the experiment. The cells were pre-treated with ES936 (5 μmol/L) for 45 min and incubated with 20 μmol/L of βL for 5 min. (*F*) bEnd.3 cells were pre-treated with LY (25 μmol/L), Wortmannin (Wort; 100 nmol/L), or KN-93 (KN; 20 μmol/L) for 45 min and then stimulated with 20 μmol/L of βL for 5 min. Immunoblot analyses were performed using the indicated antibodies. Representative blots from three independent experiments are shown.

eNOS staining localized to the aortic endothelium was observed after 6 weeks of βL treatment (*Figure 6B*). These data indicate that eNOS phosphorylation and protein expression are all stimulated by βL treatment, resulting in increased eNOS activity in the aorta and reduced SHR BP.

4. Discussion

In this study, we found that βL treatment increased cytoplasmic NAD^+/NADH ratio through NQO1 activation resulting in reduced BP and vascular tension in SHR. These effects were completely blocked by eNOS inhibition with L-NAME (*Figures 1 and 2*). This data strongly suggested that the hypotensive effect of βL resulted from endothelial eNOS activity modulation. As we were interested in endothelial cell eNOS modulation by βL, we measured cellular NAD^+/NADH ratio in endothelial cells. As expected, βL treatment significantly increased total NAD^+/NADH ratio (*Figure 1D*) and the cytoplasmic pyruvate/lactate ratio indicating free NAD^+/NADH ratio[28] (*Figure 1E*) in endothelial cells, and these results were considered a consequence of βL-induced NQO1 activation. Additionally, eNOS phosphorylation was also relatively decreased in 6 weeks βL-treated SHR, which have showed reduced BP (*Figure 6A*). From these results, it was assumed that βL-induced increase in cellular

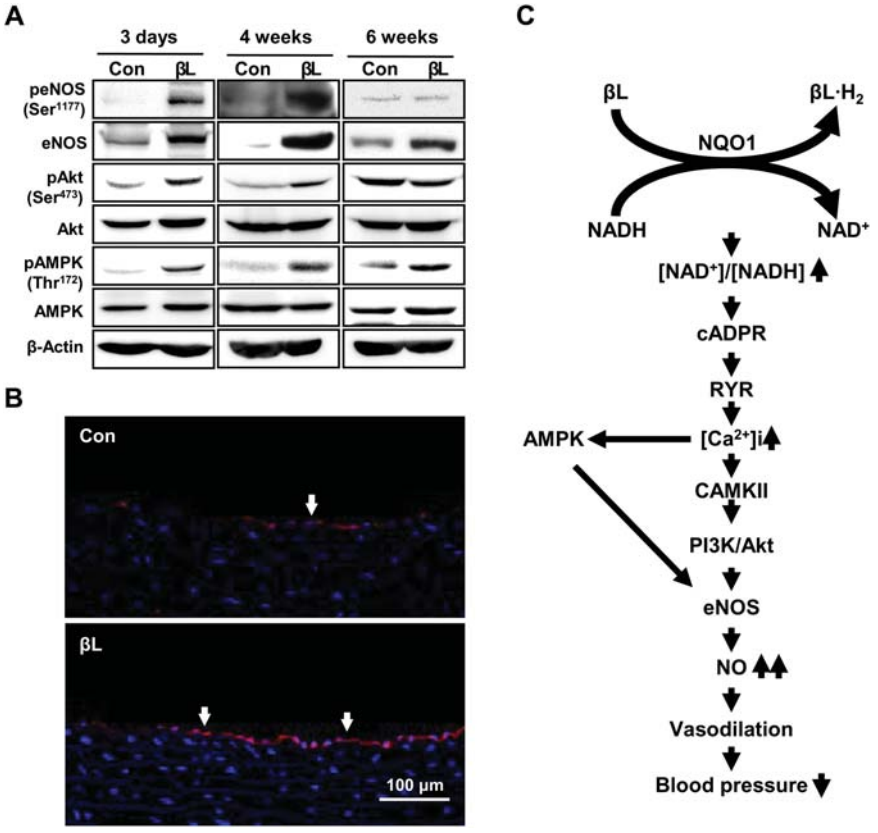

Figure 6 βL activates eNOS in the aorta of SHR. The SHR were divided into three groups containing three to six animals. 0.12% βL (wt/wt) was added to the regular chow diet for 3 days, 4 weeks, and 6 weeks. The SHR aortas were excised and pooled. (*A*) Western blot analyses were performed using indicated antibodies for total aortic protein according to the manufacturer's protocol. Representative blots from three independent experiments are shown. (*B*) The aortas of SHR treated with βL for 6 weeks were excised. Aortic eNOS protein was detected using an anti-eNOS antibody and visualized with red fluorescence. The arrow indicates the aortic endothelium. Pictures are shown at ×200 magnification with a confocal microscope. Control group, $n = 3–5$; βL group, $n = 3–6$. (*C*) Model for the BP regulation mechanism of βL.

NAD^+/NADH ratio ameliorated hypertension through vascular tone control via eNOS modulation under hypertensive stress conditions. In current study, meanwhile, eNOS is a downstream protein of NQO1, and L-NAME is a specific inhibitor of eNOS. Because we thought that L-NAME might not affect the cellular NAD^+/NADH ratio regulated by NQO1, βL, and L-NAME co-treated group was excluded in cellular NAD^+/NADH ratio analysis *in vitro*.

In general, vascular endothelial cells are ubiquitous, multifunctional, and play a crucial role in regulating BP through the control of vascular tone and homoeostasis.[6,31] BP is mediated by changes in the generation and release of the vasodilator NO. This occurs in response to various stimuli. eNOS-Ser^{1177} phosphorylation through the PI3K-dependent Akt and maintenance of eNOS protein expression by AMPK are indispensable for eNOS activation.[11–13] In the present study, βL was shown to activate eNOS-Ser^{1177} phosphorylation along with the active phosphorylation of Akt (*Figures 3*, *4*, and *6*). The AMPK inhibitor compound C did not block this phenomenon. However, LY249002 and Wortmannin (PI3K inhibitors) inhibited this eNOS phosphorylation (*Figures 4C* and *5F*). Furthermore, βL increased eNOS protein levels through AMPK phosphorylation in all measured time points in βL-treated SHR aortic tissues (*Figure 6*). The eNOS protein levels were decreased by AMPK inhibition with dnAMPKα in bEnd.3 cells (*Figure 4D* and *E*). Based on these results, it is assumed that the hypotensive effect of βL is due to the synergic effect of AMPK on eNOS protein expression and PI3K/Akt on eNOS phosphorylation.

Next, we explored the upstream mediator of eNOS phosphorylation following βL-activation of NQO1 in bEnd.3 cells. A large number of endothelial functions including, notably, eNOS activity, depend on $[Ca^{2+}]$i changes[15] to varying extents. cADPR generated from NAD^+ by ADP-ribosyl cyclase was reported to participate in intracellular Ca^{2+} oscillations in a RyRs-dependent manner in endothelial cells.[16,32,33] In the present study, intracellular calcium fluxes were increased by endothelial cell βL treatment. This βL-stimulated increase in $[Ca^{2+}]$i was completely inhibited by the intracellular calcium chelator (BABTA-AM) and the RyRs antagonist (8-Br-cADPR), but not by the extracellular calcium chelator (EGTA) (*Figure 5A*). Moreover, inhibition of $[Ca^{2+}]$i was observed following NQO1 inhibitor (dicoumarol and ES936) treatment (*Figure 5B*). In parallel with the inhibition of $[Ca^{2+}]$i, βL-induced active phosphorylation of PI3K/Akt-eNOS and AMPK was inhibited (*Figure 5C*, *D*, and *E*). These results indicate that the elevated cytoplasmic NAD^+/NADH ratio resulting from βL-induced NQO1 activation can increase $[Ca^{2+}]$i via RyR-mediated Ca^{2+} release. Moreover, the βL-induced elevation of $[Ca^{2+}]$i promotes eNOS activity through PI3K/Akt and AMPK phosphorylation. Resveratrol was previously

reported to increase cytosolic Ca^{2+} levels and activate AMPK by the calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ).[34] PI3K is positively regulated by Ca^{2+} via CaMKII.[35] Our results show that increased PI3K/Akt-eNOS phosphorylation by βL was completely abolished by CaMKII inhibition in this study (*Figure 5F*). Taken together with previous studies,[34,35] our results provide strong evidence suggesting that βL-induced phosphorylation of PI3K/Akt and AMPK is mediated by Ca^{2+}-induced activation of CaMKII or CaMKKβ.

In conclusion, this is the first study demonstrating that NQO1 activation by βL leads to decreased BP in a hypertensive animal model. As summarized in *Figure 6C*, the hypotensive effect of βL is attributable to NO-mediated vascular relaxation via aortic eNOS activation. This is mediated by active phosphorylation of PI3K/Akt and AMPK through $[Ca^{2+}]i$ elevation, which is mediated by modulation of cytoplasmic NAD^+/NADH ratio via NQO1 activation. Previous studies reported that calorie restriction and exercise have beneficial effects on BP in hypertensive patients during clinical trials.[36,37] In addition, calorie restriction and exercise were reported to lead to an increase in NAD^+/NADH ratio.[38–40] Therefore, modulation of cellular NAD^+/NADH ratio might be a new therapeutic approach for hypertension.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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