NAD(P)H:Quinone Oxidoreductase 1 Activation Reduces Blood Pressure Through Regulation of Endothelial Nitric Oxide Synthase Acetylation in Spontaneously Hypertensive Rats

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BACKGROUND
Endothelial nitric oxide synthase (eNOS) is involved in blood pressure (BP) regulation through the production of nitric oxide. Sirtuin 1 (SIRT1), an NAD-dependent protein deacetylase, promotes vascular relaxation through deacetylation and activation of eNOS. β-Lapachone (βL) increases the cellular NAD+/NADH ratio by activating NAD(P)H:quinone oxidoreductase 1 (NQO1). In this study, we verified whether activation of NQO1 by βL modulates BP through regulation of eNOS acetylation in a hypertensive animal model.

METHODS
Spontaneously hypertensive rats (SHRs) and an endothelial cell line (bEnd.3 cells) were used to investigate the hypotensive effect of βL and its mechanism of action.

RESULTS
βL treatment significantly lowered the BP in SHRs, but this hypotensive effect was completely blocked by eNOS inhibition with ω-nitro-L-arginine methyl ester. In vitro studies revealed that βL activated eNOS, which was accompanied by an increased NAD+/NADH ratio. Moreover, βL significantly decreased acetylation of eNOS; however, this reduced eNOS acetylation was completely precluded by inhibition of SIRT1 in the bEnd.3 cells and in the aorta of the SHRs. Consistent with these effects, βL-induced reduction in BP was also abolished by SIRT1 inhibition in the SHRs.

CONCLUSIONS
To the best of our knowledge, this is the first study to demonstrate that eNOS acetylation can be regulated by NQO1 activation in an SIRT1-dependent manner, which is correlated with the relief of hypertension. These findings provide strong evidence that NQO1 might be a new therapeutic target for hypertension.

Keywords: blood pressure; eNOS; hypertension; NQO1; SIRT1.

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Endothelial nitric oxide synthase (eNOS), the predominant vascular nitric oxide (NO) synthase isofrom, is responsible for the majority of NO production in the vasculature and plays a key role in the control of cardiovascular homeostasis.1–2 Indeed, inhibition of endothelial function, particularly perturbation of eNOS, has been implicated in a number of cardiovascular diseases, including hypertension.3–5

The activity of eNOS is a tightly controlled event, influenced by a series of post-translational modifications that include regulation of the phosphorylation state by kinases and phosphatases, protein–protein interactions, and cofactors.6 In addition to these regulatory mechanisms, it has recently been demonstrated that eNOS activity is influenced by its acetylation status and that sirtuin 1 (SIRT1), an NAD-dependent class III protein deacetylase, plays a role in regulating endothelial NO production and endothelium-dependent vascular tone through deacetylation of eNOS.7,8

Indeed, resveratrol, a plant polyphenol that is known to stimulate SIRT1 activity, activates eNOS, improves endothelial functions, prevents blood pressure (BP) elevation, and restores vascular eNOS activity in animal models of endothelial dysfunction.9–11 NAD(P)H:quinone oxidoreductase 1 (NQO1), a homodimeric enzyme initially identified in 1958, catalyzes the oxidation of NADH to NAD+ through various quinones.12 Indeed, a significantly reduced NAD+/NADH ratio was observed in the liver and kidneys of NQO1 knockout mice.13 Several activators and substrates of NQO1 have been identified, of which β-lapachone (βL; 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione) is the most widely known14 and has been shown to increase the NAD+/NADH ratio through NQO1 activation.15–20

Recent studies have demonstrated that NQO1 activation has beneficial effects on obesity,21 arterial restenosis,16 and...
renal injuries.19–22 With respect to BP regulation, our previous research suggested that βL-induced NQO1 activation could mitigate spontaneous hypertension through activation of eNOS by increased levels of phosphorylation and coupling.15,18 Here, we report that pharmacological activation of NQO1 regulates SIRT-dependent eNOS acetylation, which correlated with eNOS activation and the relief of hypertension in an animal model.

METHODS

Materials

βL was chemically synthesized by KT&G Life Sciences Corporation/R&D Center (Suwon, Korea). ω-Nitro-L-arginine methyl ester (L-NAME), resveratrol, nicotinamide, and sirtinol were purchased from Sigma Chemical (St Louis, MO). ES936 was purchased from Tocris Bioscience (Bristol, UK). Dicoumarol was purchased from Calbiochem (San Diego, CA). An anti-eNOS antibody was purchased from BD Biosciences (San Diego, CA), and an anti-acetylated-lysine antibody was purchased from Cell Signaling Technology (Danvers, MA).

Animals and experimental procedures

Twelve-week-old male spontaneously hypertensive rats (SHRs) were purchased from an established colony at the Japan SLC (Inasa Production Facility, Shizuoka, Japan). The experimental animals were assigned to 4 groups: (i) group 1 included 2 subgroups that received either a regular chow diet or a diet containing βL (0.12% wt/wt) for 3 days; (ii) group 2 included 2 subgroups that received either a regular chow diet or a diet containing βL for 4 weeks; (iii) group 3 comprised 3 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 as the βL group for the first 4 weeks followed by the addition of the eNOS inhibitor L-NAME to drinking water for 7 days during week 5 (50 mg/kg/day); and (iv) group 4 comprised 3 subgroups that received a regular chow diet, a diet containing βL alone, or a diet containing βL and sirtinol together (0.5% wt/wt). The concentration of βL provided in the diet was based on the results of a preliminary experiment. All rats were humanely killed by carbon dioxide asphyxiation. The rats were acclimatized to a 12-hour light/dark cycle at 22 ± 2°C for 2 weeks with unlimited food and water in a specific 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 and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell culture

Mouse brain endothelial (bEnd.3) cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco’s modified Eagle medium (Hyclone Laboratory, Logan, UT), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere (5% carbon dioxide/95% air) at 37°C.

Measurement of BP

Systolic BP (SBP) and diastolic BP (DBP) were measured weekly in all animals after treatment. The rats were preheated for 15–20 minutes at 39°C in a warming box to dilate their tail blood vessels, and their BP was then measured using the noninvasive tail-cuff method in a restrainer heated electrically to 39°C. The animals were pre-exposed to the apparatus (Softron BP-98A, Softron, Tokyo, Japan; or CODA-HT4; Kent Scientific, San Diego, CA) at least 5 times before measurements were taken.

Measurement of eNOS activity

The conversion of [14C]L-arginine to [14C]L-citrulline was used to assess eNOS activity in cultured bEnd.3 cells and aorta of SHRs using the NOS Activity Assay Kit (Cayman Chemicals, Ann Arbor, MI). Briefly, to prepare the total cell lysates, bEnd.3 cells were centrifuged at 5,000 × g for 2 minutes, and the cell pellet was sonicated briefly in homogenization buffer to disrupt the cells. To prepare the tissue lysates, the aortas were washed with ice-cold phosphate-buffered saline (PBS) and homogenized in ice-cold lysis buffer. The cell and aortic homogenates were centrifuged at 16,000 × g for 15 minutes at 4°C. The eNOS-containing supernatant was separated, and the samples were incubated with a reaction solution (50 mmol/L Tris-HCl, pH 7.4, 6 µmol/L tetrahydrobiopterin, 2 µmol/L flavin adenine dinucleotide, 2 µmol/L flavin mononucleotide, 1.25 mmol/L NADPH, and 0.75 µmol/L CaCl2) and 1 µCi [14C]L-arginine at 37°C for 60 minutes. The reaction was stopped by the addition of 400 µl cold stop buffer (50 mmol/L HEPES, pH 5.5, 5 µmol/L EDTA). Equilibrated resin (100 µl) was added to each reaction sample. After centrifugation, the radioactivity in the eluate was quantified. Radiolabeled [14C]L-citrulline was measured with a liquid scintillation counter (Tri-Carb 2910 TR; Perkin Elmer, Waltham, MA) and used to determine eNOS activity.

Measurement of the total NAD+/NADH ratio

The bEnd.3 cells were treated with either dimethyl sulfoxide (DMSO) or 10 µmol/L βL (suspended in DMSO). The treated cells were washed with ice-cold PBS and harvested with 80% methanol. The cells were then lysed mechanically with a 31-gauge needle and centrifuged. The supernatant was transferred to a tube and the remaining pellet was lysed again by sonication in 80% methanol and centrifuged. The supernatant was pooled into the previous tube. All procedures were performed at a temperature <4°C. Electrospray-ionization mass spectrometry was performed in positive ion mode using an MDS Sciex API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystems, Ontario, CA), followed by chromatographic separation with the Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA).
and X Terra MS C18 2.1 × 150 mm with a 3.5-µm column (Waters, Milford, MA), as previously described. 

Determination of the pyruvate/lactate ratio

The bEnd.3 cells were treated with either DMSO or 10 µmol/L βL for 15 minutes. The treated cells were washed with ice-cold PBS and quickly deproteinized in 400 µL of acid extraction buffer (1 mol/L HClO₃). The cells were then lysed by repeated sonication and centrifuged at 13,000 rpm for 10 minutes. After centrifugation, the supernatant was transferred to a tube and neutralized with 500 µL of 1 mol/L borate buffer (pH 11). The pyruvate/lactate ratio representing the free cytosolic NAD⁺/NADH ratio was measured using a d-lactate assay kit (Megazyme International, Bray, Ireland), according to the manufacturer’s instructions.

Immunoprecipitation and Western blot analysis

The rat aortas were washed with ice-cold PBS and homogenized in ice-cold lysis buffer. The cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer. Centrifugation was carried out at 13,000 × g for 15 minutes, and the amount of soluble protein was determined using the Bradford assay. Immunoprecipitation of eNOS was carried out by incubating the anti-eNOS antibody with 500 µg of the aorta lysates or cells. Acetylation of eNOS was analyzed using the anti-acetylated lysine antibody by Western blotting through standard procedures. Results were quantified by densitometry (TINA 2.09 g; Raytest Isotopenmessgeräte, Straubenhardt, Germany), and the ratio was determined in arbitrary units.

Statistical analysis

Numerical data are presented as the mean ± SEM. Comparisons between groups were performed using a two-tailed Student t test or one-way analysis of variance. The threshold of significance was set at P < 0.05.

RESULTS

βL decreases BP in SHRs and increases eNOS activity in endothelial cells

To identify whether βL shows hypotensive effect through eNOS dependently, SHRs received βL in the diet for 6 weeks (βL group) or received the same diet as the βL group for the first 4 weeks followed by L-NAME cotreatment for 7 days during week 5 (Figure 1a). As a result of βL supplementation in SHRs, the SBP and DBP were significantly reduced in both the βL and βL+L-NAME groups (Figure 1b,c) when compared with the control group at 4 weeks post-treatment. However, when an additional week of eNOS inhibition by L-NAME treatment was administered, the BP-lowering effect of βL on the βL+L-NAME group was blocked, and the SBP and DBP rebounded to the levels of the control group. This elevated BP in the βL+L-NAME group was reversed by cessation of the L-NAME treatment for 1 week. By contrast, the lowered SBP level was maintained in the βL-only group (Figure 1b,c). To investigate whether βL directly increases eNOS enzyme activity, the production of [¹⁴C] l-citrulline was measured in bEnd.3 cells treated with βL. Within 15 minutes of βL treatment, [¹⁴C]l-citrulline production was significantly increased, indicating an increase in eNOS activity (Figure 1d).

βL increases the NAD⁺/NADH ratio and stimulates NQO1-SIRT1–dependent eNOS deacetylation in endothelial cells

Previously it was shown that βL increases the NAD⁺/NADH ratio through NQO1 activation. Accordingly, we used bEnd.3 cells to investigate whether βL could increase the cellular NAD⁺/NADH ratio through NQO1 activation in endothelial cells. The total cellular NAD⁺/NADH ratio was significantly increased in βL-treated bEnd.3 cells (Figure 2a). The pyruvate/lactate ratio, representing the free cytosolic NAD⁺/NADH, was also significantly increased by βL treatment (Figure 2b). SIRT1 activity has been attributed to changes in the NAD⁺ abundance and NAD⁺/NADH ratio, and SIRT1-dependent deacetylation is an eNOS activation mechanism. Therefore, we assessed the level of eNOS acetylation after βL treatment in bEnd.3 cells. As shown in Figure 2c, βL treatment significantly decreased acetylation of eNOS compared with the control group and was comparable with that observed by resveratrol-induced SIRT1 activation. Furthermore, the reduced eNOS acetylation resulting from βL treatment was completely abolished by inhibition of SIRT1 with nicotinamide or inhibition of NQO1 with dicoumarol and ES936 (Figure 2d,e).

βL stimulates SIRT1-mediated eNOS deacetylation in the aorta of SHRs

We sought to ascertain whether βL-induced eNOS deacetylation could be reproduced in vivo using the aortic tissues of βL-treated SHRs. Aortic eNOS acetylation was significantly decreased after 3 days of βL treatment, compared with the same time point in the control group, and these effects were sustained during the 4 weeks of βL treatment (Figure 3a). To identify whether βL-induced aortic eNOS deacetylation was also mediated by SIRT1, we treated SHRs with either βL alone or cotreated them with sirtinol, a SIRT1 inhibitor, for 6 days. In accordance with the in vitro result shown in Figure 4b, SIRT1 inhibition with sirtinol entirely inhibited the effect of βL on eNOS deacetylation in the aorta of SHRs (Figure 3b). 

eNOS activation-related hypotensive effect of βL is partially suppressed by inhibition of SIRT1

To identify whether eNOS deacetylation by SIRT1 regulates eNOS activation and the BP-lowering effect of βL, we measured aortic eNOS activity and BP in SHRs treated with either βL alone or cotreated with sirtinol for 6 days. As shown in Figure 4a–c, βL treatment drastically elevated aortic eNOS activity and lowered the SBP and DBP levels in the βL group by 6 days after treatment compared with the control group. Intriguingly, although SIRT1 inhibition by sirtinol treatment abolished eNOS activation and the BP-lowering effects of βL, it was not completely blocked.
DISCUSSION

We recently demonstrated that modulation of the NAD+/NADH ratio by NQO1 activation could regulate eNOS activity by two mechanisms: (i) regulation of eNOS phosphorylation and protein levels through the phosphoinositide 3-kinase/Akt/5′ AMP-activated kinase (AMPK) pathway\textsuperscript{17} and (ii) eNOS coupling regulation by elevation of the GTP cyclohydrolase-1 protein level (GTPCH-1).\textsuperscript{18} This study investigated a further mechanism of eNOS activation by NQO1 and found that NQO1 activation regulates eNOS acetylation in an SIRT1-dependent manner.

It has been reported that SIRT1 binds to eNOS and directly contributes to its activation through deacetylation at lysine residues 496 and 506 in the calmodulin-binding domain.\textsuperscript{7}
However, a deacetylase-defective mutant of SIRT1 decreased NO bioavailability and attenuated endothelium-dependent vasodilation, which could be attributed to enhanced acetylation of eNOS. SIRT1 activity is known to be mainly regulated by the ratio of NAD$^+$/NADH. This study and other previous studies have shown that NQO1 activation by βL significantly increased the NAD$^+$/NADH ratio and affected SIRT1-dependent regulation of protein acetylation in endothelial cells. In this study, the increased NAD$^+$/NADH ratio by NQO1 activation stimulated direct SIRT1-dependent eNOS deacetylation and regulated eNOS activity in endothelial cells. Aortic eNOS also showed dramatically reduced acetylation and significantly increased activity with βL treatment. SIRT1 inhibition with sirtinol cotreatment completely or partially abolished the effect of βL on eNOS deacetylation or eNOS activation, respectively. These results revealed that NQO1 activation by βL increases SIRT1-dependent eNOS deacetylation and can regulate eNOS activity in the aorta of SHR.

As aforementioned, there are several known regulatory mechanisms for eNOS activity. Among them, phosphorylation of eNOS at serine 1177, which is close to the carboxy-terminal, is a critical requirement for eNOS activation. Akt (also known as protein kinase B, PKB) is the major kinase for this phosphorylation. AMPK also plays an important role in eNOS regulation by increasing the level of tetrahydrobiopterin, which is an essential cofactor for dimerization and activation of eNOS. In accordance with these 2 regulatory mechanisms, we previously reported that βL-mediated activation of NQO1 could modulate eNOS activity through both PKB/Akt-dependent eNOS phosphorylation at serine 1177 and AMPK-dependent eNOS coupling. In this study, NQO1 activation increased eNOS activity and lowered the BP in SHR, which was abolished by eNOS

Figure 2. β-Lapachone (βL) induces NADH oxidation and decreases acetylation of endothelial nitric oxide synthase (eNOS) in endothelial cells. (a and b) bEnd.3 cells were treated with 10 μmol/L βL for 15 minutes. (a) The NAD$^+$/NADH ratio was calculated from the levels of total NAD$^+$ and total NADH measured by high-performance liquid chromatography tandem mass spectrometry analysis. (b) Cells were harvested with 1 mol/L perchloric acid. The supernatant was used for pyruvate and lactate measurements. (c–e) bEnd.3 cells were stimulated with 10 μmol/L βL or 100 μmol/L resveratrol (Res) for 15 minutes or were pre-treated with 5 mmol/L nicotinamide (NAM), 10 μmol/L dicoumarol (Dicou), or 5 μmol/L ES936 for 1 hour and stimulated with 10 μmol/L βL for 15 minutes. The treated cells were immunoprecipitated with an eNOS antibody, and eNOS acetylation was determined using an acetylated-lysine antibody. The graph shows the results of densitometric analysis of acetylated-lysine (Acetyl Lys) relative to the total eNOS. Grouped quantitative data are presented as means ± SEMs from a minimum of 3 independent experiments. Significance was measured with a 2-tailed Student t test or 1-way analysis of variance. *$P<0.05$, **$P<0.01$, ***$P<0.001$, for the experimental group compared with the control group; $^#P<0.05$, $^##P<0.01$, for the experimental group compared with the βL group.
inhibition with L-NAME cotreatment. This suggested that eNOS is a key mediator of BP modulation by NQO1 activation. However, SIRT1 inhibition with sirtinol did not completely block the eNOS-mediated BP-lowering effect of βL, although it did suppress βL-induced eNOS deacetylation in the aorta of SHRs. Meanwhile, coupling of eNOS could be regulated in part by SIRT1-dependent LKB1 deacetylation through modulation of AMPK-mediated GTPCH-1 preservation. Therefore, SIRT1 inhibition by sirtinol might also influence on eNOS activity through partial suppression of eNOS coupling. Collectively, these results suggest that eNOS-mediated BP regulation by NQO1 activation is not entirely dependent on eNOS deacetylation and must also be modulated by another mechanism such as phosphorylation and/or dimerization of eNOS.

It has also been reported that eNOS activity is synergistically regulated by active phosphorylation and deacetylation in shear stress-exposed aorta. Phosphorylation of eNOS enhances its affinity toward SIRT1, enabling eNOS deacetylation. In view of eNOS modulation by βL, NQO1 activation...
has been shown to enhance both active phosphorylation and deacetylation of eNOS. Therefore, NQO1 activation by βL might induce a synergism between phosphorylation and deacetylation of eNOS and could thus augment eNOS activity.

In conclusion, we have shown that NQO1 activation by βL elicits a hypotensive effect, at least in part, through regulation of eNOS acetylation and as a result of cellular NAD+/NADH ratio-modulated SIRT1 activation. Although a combination of medications is used for hypertension therapy,30,31 the development of novel and expeditious targets, which could afford multiple effects for new therapeutic interventions, is important for the diversification of long-term combinatorial prescriptions. The experimental results from our current and previous studies17,18,32 strongly support NQO1 as a new therapeutic target for hypertension treatment that could also provide diversification in current hypertension medications.

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DISCLOSURE

The authors declared no conflict of interest.

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