NQO1 activation regulates angiotensin-converting enzyme shedding in spontaneously hypertensive rats

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Aims
Angiotensin-converting enzyme (ACE) plays a key role in blood pressure (BP) homeostasis via regulation of angiotensin II. Active ACE ectodomain is enzymatically cleaved and released into body fluids, including plasma, and elevated plasma ACE levels are associated with increased BP. β-lapachone (βL) has been shown to increase cellular NAD+/NADH ratio via activation of NAD(P)H:quinone oxidoreductase 1 (NQO1). In this study, we evaluated whether NQO1 activation by βL modulates BP through regulation of ACE shedding in an animal model of hypertension.

Methods and results
Spontaneously hypertensive rats (SHR) and a human ACE-overexpressing rat lung microvascular endothelial cell line (RLMVEC-hACE) were used to investigate the mechanism by which βL exerts a hypotensive effect. In vitro studies revealed that βL significantly increased intracellular Ca²⁺ ([Ca²⁺]i) levels and CaMKII Thr²⁸⁶ phosphorylation, followed by diminished ACE cleavage secretion into culture media. Inhibition of βL-induced [Ca²⁺]i level changes through intracellular Ca²⁺ chelation, Nqo1-specific siRNA or ryanodine receptor blockade abolished not only βL-induced increase in [Ca²⁺]i levels and CaMKII phosphorylation, but also βL-mediated decrease in ACE shedding. The effect of βL on ACE shedding was also blocked by inhibition of CaMKII. In SHR, βL reduced BP following increase of CaMKII Thr²⁸⁶ phosphorylation in the lung and decrease of ACE activity and angiotensin II levels in plasma.

Conclusion
This is the first study demonstrating that ACE shedding is regulated by NQO1 activation, which is possibly correlated with relieving hypertension in SHR. These findings provide strong evidence suggesting that NQO1 might be a new target for ACE modulation and BP control.

Keywords
Angiotensin-converting enzyme • Blood pressure • CaMKII • NQO1

1. Introduction
Angiotensin-converting enzyme (ACE) is a central component of the renin–angiotensin system (RAS), which regulates blood pressure (BP) and electrolyte balance. Angiotensin-converting enzyme (ACE) is a type I ectoprotein anchored in the plasma membrane of endothelial cells and comprises a short cytoplasmic domain, a transmembrane domain, and a long extracellular domain containing the catalytic active site. Cleavage of the membrane proximal region of ACE allows the enzymatically active extracellular domain to be released into body fluids, including plasma, and both the membrane bound and soluble forms of ACE are active. Several human studies have reported that an increase in plasma ACE levels and activity may increase BP through modulating levels of angiotensin II, which is a main effector of the RAS. Therefore, the regulation of ACE shedding and the

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2.2 Cell culture

ACE expression on the plasma membrane of primary culture endothelial cells decreases with propagation and passage. Therefore, to study the effect of βL on endothelial ACE shedding in vitro, we used a human ACE-overexpressing rat lung microvascular endothelial cell line (RLMVEC-hACE) previously described by Balyasnikova et al.

2.3 Statistical analysis

Numerical data are presented as the mean ± SEM. Comparisons between groups were performed using a two-tailed Student’s t-test, one-way ANOVA, or two-way ANOVA. The threshold of significance was set at \( P < 0.05 \).

3. Results

3.1 βL activates CaMKII via Thr\(^{286}\) phosphorylation and inhibits cleavage secretion of ACE

First, the capacity of βL to induce phosphorylation of CaMKII at Thr\(^{286}\), a catalytically active phosphorylation site, was investigated in RLMVEC-hACE. As shown in Figure 1A and D, βL significantly elevated CaMKII Thr\(^{286}\) phosphorylation in a concentration- and time-dependent manner. It has been reported that CaMKII regulates cleavage-mediated secretion of ACE. In RLMVEC-hACE, ACE is constitutively cleaved and secreted at a basal level. As ACE is cleaved and released from the cell membrane, it is decreased in cell lysate and increased in the media. As shown in Figure 1B and E, ACE protein levels in RLMVEC-hACE were increased by βL treatment compared with control group in a concentration- and time-dependent manner. In accordance with an increase in ACE protein levels in cell lysates, βL treatment significantly increased ACE levels in the culture media in a concentration- and time-dependent manner (Figure 1C and F). On the basis of these results, it appears that βL suppresses ACE shedding by promoting phosphorylation of CaMKII at Thr\(^{286}\).

3.2 βL-induced inhibition of ACE shedding is mediated by NQO1-dependent CaMKII activation

Inhibition of CaMKII activity in RLMVEC-hACE was performed to confirm the relationship between Thr\(^{286}\) phosphorylation of CaMKII and cleavage-mediated release of ACE. As shown in Figure 2A, βL-induced CaMKII Thr\(^{286}\) phosphorylation was completely blocked by KN-93 treatment. Intriguingly, this KN-93-mediated suppression of CaMKII activation inhibited the effects of βL on ACE protein levels in RLMVEC-hACE and the culture media (Figure 2B and C). To determine whether these effects were NQO1-dependent, endogenous NQO1 was suppressed by transfecting RLMVEC-hACE with Nqo1 siRNA. Inhibition of endogenous NQO1 by Nqo1 siRNA completely inhibited the effects of βL on Thr\(^{286}\) phosphorylation of CaMKII (Figure 2D). In line with this inactivation of CaMKII, Nqo1 siRNA also significantly diminished the effects of βL on ACE release from the surface of RLMVEC-hACE (Figure 2E and F). These data indicate that the inhibitory effect of βL on ACE shedding is regulated by Thr\(^{286}\) phosphorylation of CaMKII via an NQO1-dependent mechanism.

3.3 ACE shedding is diminished by βL through an NQO1-dependent increase in intracellular calcium

Ca\(^{2+}\) is an important regulator of CaMKII activity. In the presence of Ca\(^{2+}\)/calmodulin, CaMKII can autophosphorylate at Thr\(^{287}\) and become constitutively active. To investigate whether βL influences [Ca\(^{2+}\)]\(i\), intracellular calcium fluxes were estimated using a fluo-4/AM-based assay after βL treatment in vitro. βL treatment resulted in increased [Ca\(^{2+}\)]\(i\) levels within 10 s, and this [Ca\(^{2+}\)]\(i\) level was
maintained for 5 min (Figure 3A). However, the increase in [Ca²⁺]i resulting from βL treatment was completely abolished by blockade of NQO1 with ES936 and/or dicoumarol, as well as by intracellular Ca²⁺ chelation with BAPTA-AM (Figure 3A and B). With regard to [Ca²⁺]i regulation mechanism by NQO1 activation, cyclic adenosine diphosphoribose (cADPR), a Ca²⁺ mobilizing metabolite generated from NAD⁺ by ADP-ribosyl cyclase, was reported to stimulate [Ca²⁺]i oscillations through increase of ryanodine receptor (RyR) open probability via inhibition of calstabin2 (FKBP12.6) binding to RyR. In addition, we have previously shown that NQO1 activation elevated cellular NAD⁺ level, which is the main source of cADPR. In the present study, inhibition of RyR2, a RyR isoform predominantly expressed in endothelial cell, with cADPR antagonist (8-Br-cADPR) completely blocked NQO1 activation-mediated elevation of [Ca²⁺]i in RLMVEC-hACE (Figure 3C). Even though CaMKII was activated by βL treatment, RyR2 phosphorylation at Ser 2814, the primary CaMKII target active phosphorylation site in RyR2, was not increased, but
and B

1.91 mmHg) by 6 weeks post-treatment, compared with the control in the implementation. Surprisingly, both SBP and DBP were significantly reduced in both SBP and DBP by 6 weeks post-treatment, compared with the control in the implementation. Surprisingly, both SBP and DBP were significantly reduced. To ascertain whether activation in the lungs of SHR has also been shown to decrease pattern (Supplementary material online, Figure S1). In regard to another [Ca^{2+}]i regulatory protein, SERCA pump activity was shown slightly increased pattern by βL treatment, but there were no statistically significant differences among all conditions (Supplementary material online, Figure S2). Therefore, it could be suggested that the increase of [Ca^{2+}]i by NQO1 activation is mediated through NAD^{+}-cADPR-dependent elevation of RyR2 open probability.

To determine whether the change in [Ca^{2+}]i induced by βL precedes CaMKII activation and ACE shedding, Thr287 phosphorylation of CaMKII in RLMVEC-hACE and the protein level of ACE in RLMVEC-ACE and the culture media were verified under the conditions described for the experiments shown in Figure 3A-C. In accordance with the change in [Ca^{2+}]i levels in the presence of NQO1 inhibition, Figure 2D-F showed that the effect of βL on CaMKII activation and cleavage-dependent release of ACE was completely inhibited. Moreover, BAPTA-AM and 8-Br-cADPR-mediated suppression of increased [Ca^{2+}]i also significantly abolished the effects of βL on CaMKII Thr286 phosphorylation and ACE shedding (Figure 4A-F). These data strongly suggest that βL-induced increases in [Ca^{2+}]i occur prior to CaMKII-mediated ACE shedding in lung endothelial cells.

3.4 βL reduces SBP and DBP in SHR

To identify whether βL-stimulated inhibition of ACE shedding has a BP-modulating effect, BP was measured in SHR after 6 weeks of βL supplementation. Surprisingly, both SBP and DBP were significantly reduced in the βL-treated group (SBP, 165.5 ± 3.13 mmHg; DBP, 105.1 ± 1.91 mmHg) by 6 weeks post-treatment, compared with the control group (SBP, 184.9 ± 3.23 mmHg; DBP, 124.0 ± 6.23 mmHg; Figure 5A and B).

3.5 βL regulates ACE release via CaMKII activation in the lungs of SHR

To ascertain whether βL-induced CaMKII Thr286 phosphorylation and ACE shedding, these conditions were reproduced in vivo and lung tissues from βL-treated SHR were examined. As shown in Figure 6A and B, CaMKII Thr286 phosphorylation and ACE protein levels were significantly increased in the lungs after 6 weeks of βL supplementation. To determine the effect of βL on ACE gene expression, the ACE mRNA levels in the lungs were measured using quantitative real-time PCR. As shown in Figure 6C, βL administration did not affect ACE gene levels in the lungs of SHR. In accordance with the levels of ACE protein in the lungs (Figure 6B), ACE activity also tended to be elevated (P = 0.075) in the lungs of the βL-treated group (Figure 6D), while the plasma ACE activity was significantly decreased by βL supplementation (Figure 6E). Finally, the plasma levels of angiotensin II were significantly reduced in the βL-treated group compared with the control group (Figure 6F). These data indicate that CaMKII-mediated inhibition of ACE shedding is stimulated by βL treatment, resulting in decreased angiotensin II levels in the plasma.

4. Discussion

The aim of the present study was to investigate whether stimulation of NQO1 by βL can alter BP via regulation of ACE cleavage from the surface of endothelial cells. In a recent study, we reported that NQO1 stimulation by βL can alter BP in SHR through activation of eNOS.23 Here, we further investigated the mechanism of the hypotensive effect of NQO1 stimulation, and found that NQO1 activation modulates plasma angiotensin II levels through ACE shedding via CaMKII activation in lung endothelial cells and the lungs of SHR.

ACE, which plays a central role in BP regulation, is a type-I membrane protein that undergoes ectodomain shedding to generate an enzymatically active soluble form. The release of ACE from the cell membrane is known to be a regulated process.10,11,20,31 High levels of plasma ACE, some cases of which are linked to ACE gene polymorphism, have been shown in several cardiovascular disease conditions, including hypertension.12–15,32–34 While there is evidence for association between ACE genotype and plasma ACE activity, experimental data obtained in segregating rats have been shown that variant of ACE gene determines plasma ACE activity but exhibits no direct effect on BP.35 Although the data on the relation between plasma ACE activity and arterial hypertension are still ambiguous, a thorough understanding of the cleavage-dependent release process of ACE is particularly important. It has been reported that ACE shedding is stimulated by protein kinase C activation,36 tyrosine phosphatase inhibition,37 or blocking casein kinase 2 activity,38,39 and that it is inhibited by suppression of a specific class of metalloproteinases.11 In addition to these cellular proteins, ACE ectodomain shedding was increased by inhibition of CaMKII, which is a ubiquitous Ca^{2+}/calmodulin-dependent enzyme involved in various Ca^{2+}-mediated mechanisms,7 in endothelial cells,20 suggesting

Figure 3 βL increases intracellular calcium in cADPR-RyR dependent manner. (A–C) [Ca^{2+}]i was measured using the fluorescent dye (fluo-4/AM) and confocal microscopy in RLMVEC-hACE. Cells were pre-treated with ES936 (ES; 20 μmol/L), dicoumarol (Dicou; 10 μmol/L), BAPTA-AM (BAPTA; 20 μmol/L), or 8-Bromo-cADPR (Br-cADPR; 100 μmol/L) for 45 min. During scanning, 20 μmol/L of βL was added and green fluorescence was detected every second for 5 min. Increases in relative fluorescence were quantified using the regions of interest (ROI) tool. A representative graph from three independent experiments is shown.
that CaMKII is involved in the ACE shedding process. We demonstrated that an increased cellular NAD+/NADH ratio resulting from NQO1 activation can elevate [Ca$^{2+}$]i via cADPR-RyR2-dependent Ca$^{2+}$ release from the endoplasmic reticulum in mouse brain endothelial cells.\(^{23}\) In the present study, an NQO1-mediated increase in [Ca$^{2+}$]i was also shown in cADPR-RyR2-dependent manner, which resulted in CaMKII activation via phosphorylation of Thr$^{286}$ in RLMVEC-hACE. In accordance with the relationship between CaMKII and ACE shedding, elevated phosphorylation of CaMKII Thr$^{286}$ by NQO1 activation showed a negative correlation with ACE shedding in RLMVEC-hACE and the lungs of SHR, and treatment with KN-93, a potent CaMKII inhibitor, completely blocked the inhibitory effect of BL on ACE cleavage secretion. Moreover, the NQO1-dependent nature of these BL-induced effects is further supported by data obtained following NQO1 knockdown, intracellular Ca$^{2+}$ chelation and RyR inhibition studies. All of these results strongly suggest that NQO1 stimulation promotes CaMKII activity by regulating Thr$^{286}$ phosphorylation, resulting in the suppression of ACE shedding in endothelial cells.

Circulating angiotensin II is the main effector of RAS and is involved in the regulation of BP as well as fluid and electrolyte balance.\(^5\)\(^{,}\)\(^6\) Some studies have hypothesized that local production of angiotensin II by endothelial-bound ACE is a critical feature of BP regulation,\(^4\)\(^1\)\(^,\)\(^2\) and it has been reported that mice with a genetic block of ACE have a lower SBP than control animals.\(^5\)\(^\cdot\)\(^7\) However, these previous studies which are used whole-body ACE knock-out mice have less clear points; whether this system functions as a systemic endocrine RAS network or whether it functions as a series of local autocrine/paracrine RAS networks within various organs. The mice, not producing vascular ACE but expressing the enzyme on the cell membrane of hepatocyte, maintained normal BP and they were not hypotensive unless stressed by salt-free diet.\(^4\)\(^3\)\(^,\)\(^4\)\(^4\) Based on these previous data, it could be suggested that circulating plasma ACE secreted from other tissues can at least partially compensate endothelial ACE deficiency-induced dysregulation of BP. Meanwhile, several human studies have reported that hypertension is correlated with the level of circulating soluble ACE activity. An insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene, identified in 1990 by Rigat et al.,\(^4\) is now known to be significantly associated with elevated plasma ACE levels in men.\(^4\) Three large prospective human studies on the ACE I/D polymorphism have shown that the ACE D/D geno-type tended not only to be hypertensive, but also to have higher circulating levels of active ACE.\(^3\)\(^\cdot\)\(^15\) Women with pregnancy-induced hypertension also present elevated plasma ACE levels compared with normotensive women.\(^12\) In the current study, although Ace gene...
expression was not changed by \(\beta\text{L}\) treatment in the lungs of SHR, increased levels of ACE protein were observed in the lungs of \(\beta\text{L}\)-treated SHR due to the decreased amount of released ACE. As a consequence, \(\beta\text{L}\)-treated SHR had significantly reduced circulating levels of ACE and plasma angiotensin II, resulting in decreased BP. Collectively, and in accordance with previous reports, our results demonstrate a positive correlation between increased plasma ACE activity and hypertension.

Plasma ACE levels have recently been suggested to represent a risk factor for cardiovascular diseases (CVD), including myocardial infarction and coronary artery disease.\(^{33,34}\) Indeed, elevated plasma ACE activity determined less than 4 h after the onset of myocardial infarction in humans has been suggested to be a significant predictor of the development of left-ventricular dilation 1 year after infarction.\(^{47}\) Several studies have revealed that obese individuals show not only a higher rate of CVD mortality and hypertension, but also elevated plasma ACE levels compared with subjects in a normal weight group.\(^{48-52}\) Intriguingly, dietary weight loss induced by caloric restriction (CR) decreased circulating ACE levels accompanied by a decrease in BP in obese adults.\(^{53}\) During clinical trials, CR also has been shown to have beneficial effects on patients with hypertension, another major risk factor for CVD.\(^{54-56}\) CR has also been reported to lead to an elevated cellular NAD\(^+/\)NADH ratio, similar to the effect of \(\beta\text{L}\)-induced NQO1 stimulation.\(^{57}\) Considering that NQO1 stimulation-mediated improvement of hypertension and reduction of circulating ACE activity result from NAD\(^+/\)NADH ratio-dependent regulation of ACE shedding, modulation of NQO1 activity might mitigate CVD-induced tissue damage similar to the beneficial effects of CR observed in obese hypertensive patients.

With respect to myocardopathy, a CVD-related pathophysiological phenotype that occurs under chronic hypertensive conditions, NQO1 activation by \(\beta\text{L}\) significantly diminished plasma creatine kinase levels, a well-known marker of heart injury (Supplementary material online, Figure S3).\(^{58}\) In this context, it appears that NQO1 stimulation may protect against cardiac damage resulting from chronic hypertensive stress.

In conclusion, the current study is the first to demonstrate that the inhibitory effect of NQO1 activation by \(\beta\text{L}\) on ACE shedding is due to CaMKII stimulation following cADPR-RyR2-dependent \([\text{Ca}^{2+}]_i\) increase, and could be attributable to the hypotensive effect of \(\beta\text{L}\) in a hypertensive animal model. These findings provide new insight into the mechanism of ACE shedding regulation by NQO1 activation, and its possible correlation with BP control.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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