Redox-Sensitive Endoplasmic Reticulum Stress and Autophagy at Rostral Ventrolateral Medulla Contribute to Hypertension in Spontaneously Hypertensive Rats

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Abstract—Perturbations of proper functions of the endoplasmic reticulum (ER) cause accumulation of misfolded or unfolded proteins in the cell, creating a condition known as ER stress. Prolonged ER stress has been implicated in hypertension. Oxidative stress in the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of vasomotor tone reside, plays a pivotal role in neurogenic hypertension. This study aimed to evaluate the contribution of ER stress in RVLM to oxidative stress–associated hypertension and delineate the underlying molecular mechanisms. The expression of glucose-regulated protein 78 kDa and the phosphorylation of protein kinase RNA-like ER kinase–translation initiation factor α, 2 major protein markers of ER stress, were augmented in RVLM and preceded the development of hypertensive phenotype in spontaneously hypertensive rats. In RVLM of spontaneously hypertensive rats, stabilizing ER stress by salubrinal promoted antihypertension, and scavenging the reactive oxygen species by tempol reduced the augmented ER stress. Furthermore, induction of oxidative stress by angiotensin II induced ER stress in RVLM, and induction of ER stress by tunicamycin in RVLM induced pressor response in normotensive Wistar-Kyoto rats. Autophagy, as reflected by the expression of lysosome-associated membrane protein-2 and microtubule-associated protein 1 light chain 3-II (LC3-II), was significantly increased in RVLM of spontaneously hypertensive rats and was abrogated by salubrinal. In addition, inhibition of autophagy or silencing LC3-II gene in RVLM resulted in antihypertension in spontaneously hypertensive rats. These results suggest that redox-sensitive induction of ER stress and activation of autophagy in RVLM contribute to oxidative stress–associated neurogenic hypertension. (Hypertension. 2013;61:1270-1280.) • Online Data Supplement

Key Words: angiotensin II • autophagy • endoplasmic reticulum stress • neurogenic hypertension • oxidative stress

The endoplasmic reticulum (ER) is the cellular organelle responsible for synthesis, maturation, and trafficking of a wide range of proteins. Perturbations of proper functions of the ER cause accumulation of misfolded or unfolded proteins in the cell and create a condition known as ER stress. Cells cope with ER stress by turning on the unfolded protein response (UPR) through upregulation of ER-resident chaperones, inhibition of protein synthesis, and activation of protein degradation, resulting in elimination of the accumulated misfolded proteins in the ER. Generation of the reactive oxygen species (ROS), inflammation, and apoptosis occur when ER stress is sustained, and the UPR fails to control the level of unfolded and misfolded proteins in the ER, resulting ultimately in the deterioration of normal cellular functions. Three classes of ER stress transducers, including inositol-requiring kinase 1, protein kinase RNA-like ER kinase–translation initiation factor α (eIF2α), and transcriptional factor activating transcription factor-6, have so far been identified. All 3 sensors are maintained in an inactive state through an interaction of their N terminus with glucose-regulated protein 78 kDa (GRP78). When unfolded proteins accumulate in the ER, GRP78 is rapidly dissociated from the ER sensors to initiate the UPR.

A growing body of evidence implicates the involvement of ER stress in the pathophysiology of various diseases, including neurodegenerative disease, metabolic syndrome, and cardiovascular diseases. In patients with heart failure, the expression of GRP78 is significantly increased, and UPR is activated. In advanced atherosclerotic plaques, the presence of oxidized lipids, inflammation, and metabolic stress induces ER stress and activates UPR. ER stress is also related to cardiac damage and vascular endothelial dysfunction in angiotensin II (Ang II)–induced hypertension. Whether ER stress and UPR are causal to defects of cardiovascular phenotypes under the disease conditions, however, are not fully understood.

Similar to ER stress, oxidative stress that results from an imbalance of generation over degradation of the ROS is associated with a variety of cardiovascular diseases, including heart failure, atherosclerosis, and hypertension. Several
recent studies have demonstrated that the redox-sensitive changes in biochemical compositions, as well as architecture and functions of subcellular organelles, including the mitochondria, ER, and nucleus, underpin phenotypic trait of cardiovascular diseases. Of note is a recent demonstration that oxidative stress causes ER stress, with subsequent activation of UPR, leading to tissue damage and manifestation of myocardial dysfunction.

In the rostral ventrolateral medulla (RVLM), where sympathetic premotor neurons for the maintenance of vasomotor tone are located, emerging evidence supports a pivotal role for oxidative stress in neural mechanism of hypertension. Whether ER stress occurs in RVLM under hypertension and its relationship to oxidative stress have not been documented. The present study was undertaken to evaluate the contribution of ER stress in RVLM to oxidative stress–associated neurogenic hypertension, using spontaneously hypertensive rats (SHR) as an animal model. We also delineated the molecular mechanisms underlying ER stress–associated neurogenic hypertension.

Methods

All experimental procedures were performed in compliance with the guidelines of our institutional animal care and use committee, and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult (3, 6, 12, or 16 weeks of age) male SHR (74–336 g; n=249) or age-matched normotensive Wistar-Kyoto (WKY) rats (80–345 g; n=211) purchased from the Experimental Animal Center of the National Applied Research Laboratories, Taiwan, were used. SHR received an ER stress protector, salubrinal; a superoxide dismutase (SOD) mimetic, tempol; an autophagy inhibitor, 3-methyladenine (3-MA); bafilomycin A1 or the cisterna magna or microinjected directly into the bilateral RVLM. The doses used were modified from previous studies that validated the selectivity and effectiveness of the chemicals, or were determined in pilot studies. The key experimental procedures included measurement of mean arterial pressure (MAP) and heart rate by radiotelemetry under conscious conditions or in animals maintained under propofol (20 mg·kg−1·h−1) anesthesia; power spectral analysis of systolic blood pressure signals; determination of mRNA or protein expression by reverse transcription real-time polymerase chain reaction or Western blot in conventional SDS-PAGE or Phos-tag gel, gene silencing with small interfering RNA, immunohistochemical staining, double fluorescence staining, and quantification of tissue level of ROS in RVLM. Detailed procedures are available in the online-only Data Supplement.

Statistical Analysis

Data are expressed as mean±SEM. The statistical software SigmaStat (SPSS, Chicago, IL) was used for data analysis. One-way or 2-way ANOVA with repeated measures was used to assess group means, as appropriate, to be followed by the Scheffé multiple range test for post hoc assessment of individual means. In some cases, Student’s t test was used. P value <0.05 was considered statistically significant.

Results

Augmented ER Stress in RVLM of SHR

Compared with age-matched WKY rats (MAP, 107±4 mm Hg; n=8), the expression of 2 major protein markers of ER stress, GRP78 (Figure 1A) and phosphorylated eIF2α (p-eIF2α), determined by SDS-PAGE (Figure 1B) or Phos-tag gel (Figure S1A in the online Data Supplement), was significantly greater in RVLM, but not the ventromedial medulla (Figure S1B) of SHR (16 weeks old) with established hypertension (MAP, 172±6 mm Hg; n=8). Immunohistochemical staining also demonstrated a discernible increase in the number of GRP78- or p-eIF2α–immunoreactive cells in RVLM of SHR (Figure 1C). Moreover, GRP78 (Figure 1D) and p-eIF2α (data not shown) immunoreactivity colocalized only with neurons, but not with astrocytes or microglia.

ER Stress in RVLM Precedes the Development of Hypertension in SHR

To correlate ER stress in RVLM with the development of hypertension, temporal profiles in the expression of GRP78 and p-eIF2α in RVLM were evaluated in SHR from prehypertensive (3-week-old) to established hypertensive (16-week-old) stages. In the 6-week-old prehypertensive SHR (Figure 2A), the mRNA (Figure S2) and protein expression of GRP78 (Figure 2B) and p-eIF2α (Figure 2C) were significantly increased in RVLM. Both upregulations persisted in RVLM of 12- or 16-week-old SHR with established hypertension. In addition, the tissue level of ROS was also significantly increased in RVLM of prehypertensive SHR (3 or 6 weeks old), which was sustained in SHR with established hypertension (Figure 2D).

Persistence of ER Stress in RVLM After Peripheral Antihypertensive Treatment in SHR

To further confirm that the aberrant ER stress in RVLM is not secondary to hypertension, an angiotensin-converting enzyme inhibitor, captopril (100 mg·kg−1·day−1), or a calcium channel blocker that induces hypotension via peripheral vasodilation, amlodipine (5 mg·kg−1·day−1), was ingested orally for 7 days to lower the MAP in SHR with established hypertension (16 weeks old). On day 7 after antihypertensive treatments, when MAP in the treated SHR was normalized, the upregulated expression of GRP78 and p-eIF2α in RVLM was maintained (Figure S3). The same treatments, however, did not cause apparent decrease in MAP or expression of GRP78 and p-eIF2α in RVLM of age-matched WKY rats (Figure S3).

Reduction in ER Stress in RVLM Promotes Antihypertension in SHR

To establish a causal role for ER stress in RVLM in the hypertension phenotype of SHR, salubrinal, a compound that selectively suppresses eIF2α dephosphorylation by inhibiting ER stress-activated phosphatase complexes, was used to evoke a site-specific reduction in ER stress. Figure 3 shows that intracisternal (IC) infusion of salubrinal (3 or 12 pmol·kg−1·day−1) for 14 days promoted a sustained decrease in MAP (Figure 3A) in conscious SHR, alongside a decrease in the power density of the low-frequency component of systolic blood pressure spectrum (Figure 3B), our experimental index of sympathetic neurogenic vasomotor activity. On day 14 after the infusion, salubrinal treatment suppressed eIF2α dephosphorylation, resulting in a reduction in the expression of unphosphorylated eIF2α (Figure 3C). In contrast, IC infusion of salubrinal (12 pmol·kg−1·day−1) had no effect on...
ER Stress in RVLM Induces Pressor Response in Normotensive WKY Rats

To further confirm that site-specific induction of ER stress in RVLM induces pressor response, an ER stress inducer, tunicamycin (10 nmol), was microinjected bilaterally into RVLM of WKY rats. This treatment resulted in an increase in MAP that became significant on day 1 and lasted for ≥3 days after treatment (Figure 4A). Tunicamycin also caused a time-dependent increase in the expression of GRP78 (Figure 4B) and p-eIF2α (Figure 4C) in RVLM, but no detectable change in the tissue level of ROS (Figure 4D). Microinjection of tunicamycin into areas immediately outside the confines of RVLM resulted in no apparent change in MAP (+5.4±2.3 mm Hg; n=5), evaluated on day 3 postinjection.

Protection From Oxidative Stress Attenuates ER Stress in RVLM of SHR

To study the relationship between oxidative stress and ER stress in RVLM on hypertension in SHR, tempol (10 or 100 pmol·kg⁻¹·day⁻¹) was infused into the cisterna magna.
of conscious SHR for 7 days. This resulted in a significant decrease in the elevated expression of GRP78 (Figure 5A) or p-eIF2α (Figure 5B) and the heightened tissue level of ROS in RVLM (Figure S5A), detected on day 7 after tempol infusion. The same treatment also promoted a dose-related decrease in MAP (Figure S5B). IC infusion of salubrinal (3 or 12 pmol·kg⁻¹·day⁻¹), however, had no effect on the elevated tissue level of ROS in RVLM of SHR (Figure S5C).

**Oxidative Stress Induces ER Stress in RVLM of Normotensive WKY Rats**

We further established that oxidative stress induces ER stress in RVLM by examining the expression of GRP78 and p-eIF2α in normotensive WKY rats that were subjected to IC infusion of Ang II (500 pmol·kg⁻¹·day⁻¹), a treatment scheme that was reported to increase ROS level in RVLM and induce neurogenic hypertension. At the end of the 7-day Ang II infusion, the expression of GRP78 or p-eIF2α in RVLM was significantly increased (Figure S6). This induced upregulation of GRP78 or p-eIF2α was attenuated by tempol (100 pmol) when microinjected bilaterally into RVLM on day 7 after Ang II infusion.

**ER Stress Induces Autophagy in RVLM of SHR**

Sustained ER stress results in activation of apoptotic and autophagic signaling pathways. We therefore investigated whether the observed oxidative stress–associated ER stress activates these signaling pathways in RVLM. Compared with age-matched (16-week-old) WKY rats, the expression of lysosome-associated membrane protein-2 (LAMP-2; Figure 6A) and the ratio of microtubule-associated protein 1 LC3-II over LC3-I (Figure 6B), 2 markers of cellular autophagy, were significantly increased in RVLM of SHR. Such an increase in autophagy was blunted in SHR treated with IC infusion of salubrinal (12 pmol·kg⁻¹·day⁻¹) or a compound that inhibits initial autophagosome formation, 3-MA (5 nmol·kg⁻¹·day⁻¹) for 7 days. In contrast, the expression of cleaved-caspase 3 (Figure 6C) and caspase 12 (Figure 6D; markers for apoptosis) was comparable in RVLM of SHR and WKY rats, and was not affected by salubrinal or 3-MA treatment.

**Autophagy in RVLM Is Involved in Hypertension of SHR**

Finally, the functional significance of ER stress–associated autophagy in RVLM on hypertension of SHR is investigated.

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**Figure 2.** Mean arterial pressure (MAP; A), representative gels (inset) or densitometric analysis of protein level of glucose-regulated protein 78 kDa (GRP78; B) and total or phosphorylated eIF2α (p-eIF2α; C), or tissue level of reactive oxygen species (ROS; D) detected from rostral ventrolateral medulla in spontaneously hypertensive rats (SHR; 3, 6, 12, or 16 weeks of age) or age-matched Wistar-Kyoto (WKY) rats. Values are mean±SEM of 6 to 8 animals in each group (A) or quadruplicate analyses on samples pooled from 6 to 8 animals in each group (B–D). *P<0.05 vs age-matched WKY rats, and #P<0.05 vs SHR (3-week-old) group in the post hoc Scheffé multiple range analysis.
Microinjection bilaterally into RVLM of 3-MA (5 nmol) or bafilomycin A1 (100 nmol) evoked a significant decrease in MAP of SHR (Figure 7A). In addition, gene silencing with small interfering GRP78 (siGRP78) or LC3B (siLC3) RNA (0.5 pmol/100 nL), which effectively suppressed GRP78 or LC3-II protein expression (Figure S7) for 3 days, resulted in a long-lasting decrease in MAP (Figure 7B) and extenuation of the augmented sympathetic neurogenic vasomotor activity in SHR (Figure 7C), but not WKY rats (data not shown). Microinjection bilaterally into RVLM of 3-MA, bafilomycin A1, siGRP78, or siLC3B, however, had no effect on the elevated tissue level of ROS (Figure S8). The siGRP78 treatment also had no effect on the augmented expression of p-eIF2α in RVLM of SHR (Figure S9).

**Discussion**

The major findings of the present study are (1) RVLM neurons of SHR exhibit greater ER stress that precedes the manifestation of hypertension phenotype; (2) protection against ER stress in RVLM of SHR promotes antihypertension; (3) site-specific induction of ER stress in RVLM results in pressor response in normotensive WKY rats; (4) induction of oxidative stress evokes ER stress in RVLM of WKY rats; (5) protection against oxidative stress in RVLM reduces ER stress in SHR; (6) redox-sensitive ER stress induces autophagy in RVLM; and (7) inhibition of autophagy in RVLM decreases the augmented sympathetic neurogenic vasomotor tone and hypertension in SHR. Oxidative stress in RVLM plays an active role in neurogenic hypertension via sympathoexcitation.10,13,17 Our present results, therefore, provide evidence for ER stress and autophagy as novel cellular and molecular mechanisms underlying oxidative stress–associated neurogenic hypertension in SHR.

ER stress represents a response by cells to transient or prolonged perturbations in ER functions, in particular those related to protein synthesis, calcium regulation, and intracellular redox potential.2,4 Despite the essential and beneficial functions of UPR during transient ER stress, prolonged ER stress often leads to tissue dysfunction and disease development.2,4 This study is the first report to demonstrate that in RVLM of hypertensive SHR where oxidative stress is prominent,13,17 ER stress, characterized by the increase in GRP78 expression and phosphorylation of eIF2α, is significantly greater than the age-matched WKY rats. In addition, the induction of ER stress by tunicamycin results in the upregulation of GRP78 expression and the phosphorylation of eIF2α in a temporal profile that coincides with the tunicamycin-induced increase in MAP of WKY rats. The observations that the SOD mimetic, tempol, abrogates ER stress induced by IC infusion of Ang II and that tunicamycin induces ER stress with no apparent effect on ROS level suggest that ER stress occurs downstream to oxidative stress in RVLM. This notion receives substantiation from the results in SHR, in which the appearance of oxidative stress (3 weeks old) precedes that of ER stress (6 weeks old) in RVLM during the progression toward hypertension. In addition, treatment

**Figure 3.** Temporal changes in mean arterial pressure (MAP; A) or the power density of the low-frequency (LF) component of systolic blood pressure spectrum (B) after intracisternal infusion of salubrinal (3 or 12 pmol·kg⁻¹·day⁻¹) or 2% dimethyl sulfoxide (DMSO) (1 μL·kg⁻¹·day⁻¹) for 14 days in spontaneously hypertensive rats. Also shown are representative Phos-tag gels (inset) of phosphorylated eIF2α (p-eIF2α) or unphosphorylated (u-eIF2α) or densitometric analysis of protein level detected from rostral ventrolateral medulla on day 14 after salubrinal infusion. Values are mean±SEM of 6 to 9 animals in each group (A and B) or quadruplicate analyses on samples pooled from 4 animals in each group (C). Data in C are expressed as a percentage of u-eIF2α relative to phosphorylated eIF2α (p-eIF2α). *P<0.05 vs control or 2% DMSO group in the post hoc Scheffé multiple range analysis.
with tempol reduces ER stress in RVLM of SHR, whereas an ER stress protector, salubrinal, has no effect on the heightened tissue level of ROS. In the heart and blood vessels, a few studies have implicated ROS as a factor that induces ER stress under pathological conditions. In rats with autoimmune myocarditis, it is likely that ER stress is downstream to oxidative stress induced by cytokines, leading to cell death in the myocardium. Peroxynitrite, a potent oxidant generated by the reaction of nitric oxide with superoxide anion, is implicated in the promotion of atherosclerosis through a mechanism involving ER stress. It is noteworthy that augmented ER stress in RVLM of SHR is not secondary to peripheral hypertension. Normalization of the elevated MAP in SHR by oral intake of captopril or amiodipine did not affect the heightened ER stress in RVLM. We interpret the observation that MAP was not further elevated despite the increase in ER stress protein markers in 16-week-old SHR to suggest that a ceiling effect of ER stress exists in the pathophysiology of hypertension. The site specificity of ER stress at RVLM in the manifestation of hypertension is confirmed by observations that microinjection of tunicamycin to areas outside the confines of RVLM did not affect MAP in normotensive WKY rats. We noted that methods used for quantitative analysis of eIF2α phosphorylation are controversial; both SDS-PAGE and Phos-tag gel have been reported in the literature. Thus, we included analysis by Phos-tag gels in the present study to validate the results obtained from the conventional SDS-PAGE used in Western blot analysis.

Figure 4. Temporal changes in mean arterial pressure (MAP; A) and representative gels (inset) or densitometric analysis of protein level of glucose-regulated protein 78 kDa (GRP78; B) and total or phosphorylated eIF2α (p-eIF2α; C), or tissue level of reactive oxygen species (ROS; D) detected from rostral ventrolateral medulla (RVLM) of Wistar-Kyoto (WKY) rats after microinjection bilaterally into RVLM of tunicamycin (10 nmol) or 2% dimethyl sulfoxide (DMSO). Values are mean±SEM of 6 to 8 animals in each group (A) or quadruplicate analyses on samples pooled from 6 to 8 animals in each group (B–D). *P<0.05 vs control (C) or 2% DMSO group in the post hoc Scheffé multiple range analysis.
chronic hypertension. In human gingival fibroblasts, activation of p38 mitogen-activated protein kinase (p38MAPK) is involved in ER stress–induced adverse responses. In addition, in human breast cells, ER stress–induced autophagy is regulated by p38MAPK and extracellular signal-regulated kinase (ERK). We reported previously that phosphorylation of MAPks, including p38MAPK and ERK1/2, in RVLM contribute to the oxidative stress–associated neurogenic hypertension. Thus, MAPks may potentially be the interposing signaling molecules for ER stress to mediate the oxidative stress–induced neurogenic hypertension.

The functional role of ER stress in neural mechanism of hypertension is revealed by observations that induction of ER stress in RVLM by tunicamycin promotes hypertension in normotensive WKY rats, whereas protection against ER stress in RVLM by salubrinal causes antihypertension in SHR. Tunicamycin induces ER stress by blocking the synthesis of all N-linked glycoproteins, resulting in accumulation of misfolded or unfolded proteins in the ER. Salubrinal reduces ER stress and attenuates unfolded or misfolded protein synthesis in the ER by inhibiting eIF2α dephosphorylation, leading to stabilization of p-eIF2α processing. Our observed augmentation of GRP78 and p-eIF2α expression elicited by tunicamycin, and a decrease in unphosphorylated eIF2α induced by salubrinal, therefore, confirmed treatment efficacy in RVLM.

The present study further unveils an active role of ER stress–dependent autophagy in RVLM in neurogenic hypertension. ER stress has recently been linked to the induction of autophagy in neuronal cells. We found that in addition to the exaggerated ER stress in RVLM of SHR, the expression of autophagic markers, including LAMP-2 and LC3-II, was also augmented. The reversal of the heightened autophagy in RVLM of SHR by salubrinal further suggests a causal role of ER stress in the activation of autophagy. Functionally, we found that autophagy in RVLM is involved in neurogenic hypertension via augmentation of sympathetic neurogenic vasomotor activity. In general, autophagy is seen as a compensatory and adaptive mechanism to ER stress by providing a dynamic homeostasis of nutrients in cells via degrading and recycling misfolded proteins. For example, autophagy produced with lower doses of ER stress inducers results in protection against ischemic/reperfusion injury in heart. Nonetheless, under chronic ER stress, an excessive and uncontrolled autophagic activation can lead to the depletion of essential molecules and organelles, which triggers autophagic cell death. This form of cell death has recently been shown to play an important role in the development of ER stress–associated cardiovascular diseases. Exactly how ER stress–associated autophagy in RVLM mediates neurogenic hypertension is not clear and awaits further elucidation. The present finding that inhibition of autophagy abrogates the augmented sympathetic neurogenic vasomotor tone of SHR offers a clue. Our preliminary results further showed that under chronic ER stress, autophagosome formation was disrupted in RVLM, together with accumulation of excitatory amino acids that included glutamate and arginine. The disturbance of normal autophagic process may, thus, result in accumulation of excitatory amino acids in RVLM to induce sympathoexcitation. This speculation requires validation.

Our results implicate that a redox-sensitive mechanism underpins the augmented ER stress in RVLM of SHR. The ER is particularly rich in oxygenases and oxidases (eg, cytochrome P450s, flavin-containing monooxygenases, prolyl, and lysyl hydroxylases), many of which are located in the ER lumen. By producing superoxide as a byproduct, the activities of these enzymes are the main determinant of the luminal redox environment in the ER. As such, alterations of the cellular redox environment either in the direction of oxidation or reduction would affect protein processing, resulting in the induction of ER stress. The notion that redox-sensitive ER stress is present in RVLM is in variance with a recent study, which shows that ER stress is the source of oxidative stress in the subfornical organ in mediating Ang II–induced hypertension. Because ER stress and oxidative stress are tightly coupled mechanisms, it is conceivable that a vicious cycle orchestrated by reciprocal interactions between ER stress and oxidative stress may play a key role in neural mechanisms of...
Our results, however, indicate a minor role for apoptosis at RVLM in ER stress–associated neurogenic hypertension in SHR. We found that the expression of activated caspase 3 and caspase 12, markers of the apoptotic pathways, in RVLM is comparable between SHR and WKY rats. Moreover, their expressions were not affected by salubrinal in SHR. Activation of proapoptotic caspase 3 in RVLM was reported to mediate sympathoexcitation and hypertension in stroke-prone SHR. It is possible that different cell death signaling pathways in RVLM may participate differentially in neuronal mechanisms of hypertension under different disease conditions. We demonstrated in the present study that ER stress in RVLM of SHR is redox sensitive, and that autophagy is involved in ER stress–associated neurogenic hypertension. However, our experimental design did not allow us to assess whether ROS induces ER stress that subsequently causes autophagy in RVLM. In this regard, our preliminary results showed that IC infusion of Ang II (500 pmol·kg⁻¹·day⁻¹) for 7 days indeed increased the expression of LAMP-2 and LC3-II in RVLM of WKY rats; and this induced activation of autophagy was partially attenuated by coinfusion of salubrinal (12 pmol·kg⁻¹·day⁻¹).

One unexpected finding that seemingly counters intuitively the chaperone role of GRP78 in ER stress is the antihypertensive action after gene knockdown of GRP78 in RVLM of SHR. This finding is also at variance with a recent report, in which tissue-specific supplement of GRP78 in the subfornical organ with adenoval vector encoding GRP78 prevents the development of hypertension induced by peripheral Ang II. Apart from its beneficial functions of abrogating ER stress, GRP78 is reported recently as an obligatory component of ER stress–induced autophagy. Gene knockdown of GRP78 has been demonstrated to block ER stress–induced autophagosome formation in human cells. These results are in line with our finding that treatment with siGRP78 attenuated the augmented expression of LC3-II in RVLM of SHR. Together with our finding of an involvement of ER stress–associated autophagy in hypertension phenotype of SHR, it is conceivable that under the scenario of chronic ER stress, as in RVLM of SHR, ER stress–associated autophagy might play a predominant role in the pathogenesis of hypertension. This notion is substantiated by our observation that suppression of autophagy in RVLM by siGRP78 reduced the elevated arterial pressure in SHR.

![Representative gels (inset) or densitometric analysis of protein level of lysosome-associated membrane protein-2 (LAMP-2; A) or ratio between light chain 3 (LC3)-II and LC3-I protein level (B), or protein level of cleaved-caspase 3 (C) or 12 (D), detected from rostral ventrolateral medulla of spontaneously hypertensive rats (SHR; 16 weeks old) or age-mated Wistar-Kyoto (WKY) rats on day 7 after IC infusion of artificial cerebrospinal fluid (aCSF), salubrinal (Sal; 100 pmol·kg⁻¹·day⁻¹) or 3-methyladenine (3-MA; 5 nmol·kg⁻¹·day⁻¹) in SHR. Values are mean±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P<0.05 vs age-mated WKY rats, and #P<0.05 vs SHR aCSF group in the post hoc Scheffé multiple range analysis.](http://hyper.ahajournals.org/10.1161/HYPERTENSIONAHA.116.013971)  

![Figure 6. Representative gels (inset) or densitometric analysis of protein level of lysosome-associated membrane protein-2 (LAMP-2; A) or ratio between light chain 3 (LC3)-II and LC3-I protein level (B), or protein level of cleaved-caspase 3 (C) or 12 (D), detected from rostral ventrolateral medulla of spontaneously hypertensive rats (SHR; 16 weeks old) or age-mated Wistar-Kyoto (WKY) rats on day 7 after IC infusion of artificial cerebrospinal fluid (aCSF), salubrinal (Sal; 100 pmol·kg⁻¹·day⁻¹) or 3-methyladenine (3-MA; 5 nmol·kg⁻¹·day⁻¹) in SHR. Values are mean±SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P<0.05 vs age-mated WKY rats, and #P<0.05 vs SHR aCSF group in the post hoc Scheffé multiple range analysis.](http://hyper.ahajournals.org/10.1161/HYPERTENSIONAHA.116.013971)
We further reason that, under normotensive conditions, GRP78 supplement is required to sustain the beneficial UPR that prevents cells from exhibiting chronic ER stress and the associated hypertension. An emerging proposition from these narratives is that depending on the degree and duration of ER stress, GRP78 might play a double-edged sword role in ER stress–associated cellular responses. At an early stage of ER stress, GRP78 may act as a chaperone to abrogate ER stress. The upregulated GRP78 under chronic ER stress, however, may lead to autophagy. Our data also indicate that under chronic ER stress, a brief knockdown in GRP78 expression by small interfering RNA may not affect the UPR, because they might have already been activated. A cautionary note that arises from this proposition is that interpretation of the role of GRP78 in ER stress–associated hypertension must take into consideration the time point when it is activated or inhibited.

We recognize that it is likely that IC infusion of Ang II may also affect medullary regions other than RVLM. Nevertheless, our observation that site-specific application of tempol to RVLM blunted the increase in GRP78 and p-eIF2α expression in RVLM induced by IC infusion of Ang II lends support to a permissive role of ROS in inducing ER stress in RVLM during hypertension. It is well established that oxidative stress in the nucleus tractus solitarii, paraventricular nucleus of the hypothalamus, and circumventricular subfornical organ contributes to neurogenic hypertension, and our preliminary results showed that ER stress was detected in nucleus tractus solitarii and paraventricular nucleus during hypertension. Whether ER stress in key brain nuclei subserving blood pressure regulation may serve as a common denominator for oxidative stress–associated neurogenic hypertension is beyond the scope of the current study and warrants further investigation.

Figure 7. Temporal changes in mean arterial pressure (MAP; A and B) or power density of low-frequency (LF) component of systolic blood pressure spectrum (C) in spontaneously hypertensive rats (SHR) after microinjection bilaterally into rostral ventrolateral medulla (at time 0) of 3-methyladenine (3-MA; 5 nmol), bafilomycin A1 (BAF A1; 100 nmol; A), or intracisternal infusion of small interfering RNA against glucose-regulated protein 78 kDa (GRP78; siGRP78), light chain 3 (LC3-II; siLC3-II) or siControl (B and C). Baseline values from Wistar-Kyoto (WKY) rats are included for comparison. Values are means±SEM of 6 to 8 animals in each group. *P<0.05 vs control group, and #P<0.05 vs WKY rats in the post hoc Scheffé multiple range analysis. For clarity, symbols for statistical significance between artificial cerebrospinal fluid (aCSF) (SHR) and Control (WKY) are not shown in A, as they are the same to that of Control (SHR) vs Control (WKY).
In conclusion, the data from the present study provide evidence of a novel role for ER stress and the associated autophagy at RVLM in oxidative stress–associated neurogenic hypertension. The identification of oxidative stress–induced ER stress and the subsequent activation of autophagy may, thus, represent potential targets for development of new therapeutic strategies for the treatment of hypertension.

**Perspective**

Both prolonged ER stress and oxidative stress have been identified as the underlying pathogenic mechanisms in a number of cardiovascular disease processes, including hypertension. Our demonstration in the present study of a causal role for ER stress at RVLM in mediating oxidative stress–associated neurogenic hypertension argues for an interaction between these 2 mechanisms in the pathogenesis of hypertension. Nonetheless, the jury is still out with regard to the precise interplay between the 2 cellular processes. Speculatively, ER stress is known to initiate ROS production and redox deviation, and oxidative stress may initiate ER stress through protein oxidative modification. In addition, whether this interplay between oxidative stress and ER stress takes place in other brain areas that subserve central cardiovascular regulation also warrants investigation. ER stress in the forebrain subfornical organ was reported recently to mediate Ang II–dependent hypertension. Likewise, the role of ER stress–induced autophagy in neural mechanism of hypertension awaits further delineation. In particular, the determinants for activating the adaptive signals of the UPR to deal with ER stress against deactivating the deleterious consequences of chronic ER stress also await delineation. Much more work is needed to truly understand the role of ER stress in hypertension and to enable translating this knowledge into useful therapeutic strategies. In this regard, our finding that chemical ER chaperone promotes anti-hypertension in SHR by modulating ER stress suggests that these small molecules may be potential drug targets for regulating ER stress pathways in hypertension.

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**Disclosures**

None.

**References**

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**Novelty and Significance**

**What Is New?**

- Endoplasmic reticulum (ER) stress is augmented in spontaneously hypertensive rats, of which oxidative stress in rostral ventrolateral medulla (RVLM) is enhanced.
- Induction of oxidative stress in RVLM induces ER stress in normotensive rats.
- Protection against oxidative stress in RVLM reduces ER stress in spontaneously hypertensive rats.
- Site-specific induction of ER stress in RVLM of normotensive rats results in pressor response.
- Protection against ER stress in RVLM of spontaneously hypertensive rats promotes antihypertension.
- Redox-sensitive ER stress induces autophagy in RVLM, resulting in manifestation of hypertension in spontaneously hypertensive rats.

**What Is Relevant?**

- Oxidative stress in RVLM plays a pivotal role in neural mechanism of hypertension.
- Our study provided evidence for ER stress and autophagy to act as novel interfacing cellular and molecular mechanisms between oxidative stress in RVLM and neurogenic hypertension.

**Summary**

Redox-sensitive induction of ER stress and activation of autophagy in RVLM contribute to oxidative stress-associated neurogenic hyperten-
Redox-Sensitive Endoplasmic Reticulum Stress and Autophagy at Rostral Ventrolateral Medulla Contribute to Hypertension in Spontaneously Hypertensive Rats
Yung-Mei Chao, Ming-Derg Lai and Julie Y.H. Chan

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REDOX-SENSITIVE ENDOPLASMIC RETICULUM STRESS AND AUTOPHAGY AT ROSTRAL VENTROLATERAL MEDULLA CONTRIBUTE TO HYPERTENSION IN SHR

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Short title: ER stress in neurogenic hypertension
Expanded Methods

All experimental procedures were carried out in compliance with the guidelines of our institutional animal care and use committee and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Animals
Experiments were carried out in male prehypertensive or established hypertensive spontaneously hypertensive rats (SHR; 74-336 g, n = 232) or age-matched normotensive Wistar-Kyoto (WKY; 80-345 g, n = 192) rats purchased from the Experimental Animal Center of the National Applied Research Laboratories, Taiwan. They were housed in an AAALAC-International accredited animal facility under temperature control (24± 0.5°C) and 12-h light/dark (08:00 to 20:00) cycle. Standard laboratory rat chow (PMI Nutrition International, Brentwood, MO) and tap water were available ad libitum. All animals were allowed to acclimatize for at least 7 days before experimental manipulations.

Measurement of Arterial Pressure by Radiotelemetry
Arterial pressure (AP) and heart rate (HR) were measured in rats under conscious conditions using a radiotelemetry system (Data Sciences International, Minneapolis, MN). For the implantation of radiotelemetry receiver, rats were anesthetized with sodium pentobarbital (50 mg/kg, IP). A flexible catheter attached to a telemetry transmitter (Data Sciences International) was inserted into the abdominal aorta immediate below the renal arteries and secured in place with surgical glue. The transmitter was secured to the abdominal muscle and remained in the abdominal cavity for the duration of the experiment. The skin was closed using non-absorbable suture, and rats were returned to individual cages positioned over an RLA-3000 radiotelemetry receiver (Data Sciences International). Animals routinely received procaine penicillin (1,000 IU, IM) injection postoperatively to prevent infection. They were allowed to recover from surgery for 7 days before the commencement of AP recording. Only animals that showed progressive weight gain after the operation were used in subsequent experiments. The averaged mean AP (MAP) recorded 60 minutes every day between 14:00 and 15:00 was used as the daily value.

Power Spectral Analysis
The AP recorded via radiotelemetry was analyzed by an arterial blood pressure analyzer (Notocord, Croissy-Sur-Seine, France) to obtain systolic blood pressure (SBP) and heart rate (HR). Continuous, on-line and real-time auto-spectral analysis (Notocord) of SBP signals based on Fourier transform was used to detect temporal fluctuations in the low-frequency (LF; 0.25-0.8 Hz) band, the power density of which was used as our experimental index for sympathetic vasomotor tone.

Implantation of Osmotic Minipump
After obtaining baseline AP for at least 3 days using radiotelemetry, animals were anesthetized with pentobarbital sodium (50 mg/kg, IP) for the implantation of osmotic minipump. A midline dorsal neck incision was made, and the dura mater between the foramen magnum and C1 lamina was exposed following dissection of muscles. The dura was perforated with a 22-gauge steel needle, and a PE-10 catheter (Clay Adams, Sparks, MD) was advanced for 5 mm into the cisterna magna. Drainage of cerebrospinal fluid (CSF) from the outer end of catheter ensured patency of the implantation. The catheter was sealed to the dura with tissue glue and the incision was closed with layered sutures. The outer end of the catheter was connected to a micro-osmotic minipump (Alzet 1007D; Durect Co., Cupertino, CA), which was placed under the skin in the neck region. Animals received procaine penicillin (1,000 IU, IM) injection postoperatively, and only animals that showed progressive weight gain after the operation were used in subsequent experiments. Test agents used for
intracisternal infusion included angiotensin II (Ang II, Sigma-Aldrich, St. Louis, MO); a protein phosphatase inhibitor which reduces ER stress by maintaining eIF2α in its phosphorylated state,6 salubrinal (Sal; Merck KGaA, Darmstadt, Germany); a compound that inhibits initial autophagosome formation,3-methyladenine (3-MA; Sigma-Aldrich); or a superoxide dismutase mimetic,8 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol; Merck KGaA). Control infusion of artificial CSF (aCSF) or 2% dimethylsulfoxide (DMSO) served as the volume and vehicle control. The composition of aCSF was (mM): NaCl 117, NaHCO₃ 25, Glucose 11, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2 and NaH₂PO₄.

Microinjection of Test Agents into RVLM
In acute experiments, test agents were microinjected into RVLM in animals maintained under propofol (20 mg·kg⁻¹·h⁻¹) anesthesia, which provided satisfactory anesthetic maintenance as indicated by the absence of withdrawal reflex to hind paw pinch. Proper anesthesia was monitored by Microinjection bilaterally of the test agents into RVLM was carried out with a glass micropipette (external tip diameter: 50-80 μm) connected to a 0.5-μl Hamilton microsyringe.1,2 The stereotaxic coordinates for RVLM were: 4.5 to 5.0 mm posterior to lambda, 1.8 to 2.1 mm lateral to midline and 7.0 to 7.5 mm below dorsal surface of cerebral cortex. These coordinates were selected to cover the extent of ventrolateral medulla in which functionally identified sympathetic premotor neurons reside.9 As a routine, a total volume of 50 nL was delivered to each side of RVLM over 1-2 minutes to allow for complete diffusion of the test agents. Functional location of RVLM neurons was carried out at the beginning of each experiment by the elicitation of a transient increase in AP (15-20 mmHg) on microinjection of glutamate. Test agents used in this study included a nucleoside antibiotic that inhibits the first step in the biosynthesis of N-linked oligosaccharides in cells to induce ER stress,10 tunicamycin (Sigma-Aldrich, St. Louis, MO); salubrinal (Merck KGaA); tempol; (Merck KGaA); autophagy inhibitor which causes lysosomal dysfunction,11 bafilomycin A1 (BAF A1; Sigma-Aldrich); or L-glutamate (Sigma-Aldrich). Microinjection of aCSF or 2% DMSO (solvent for tunicamycin or salubrinal) served as the vehicle and volume control. The doses used were established in pilot studies or adopted from similar studies that used the test agents for the same purpose as in the present study.

Small Interfering RNA Expression Vectors
Validated small interfering RNA (siRNA) for GRP78 or LC3-II, and negative control siRNA were purchased from Santa-Cruz biotech. (Santa-Cruz, CA). The siRNA was microinjected into the bilateral RVLM using TurboFact™ in vivo transfection reagent (Ferments Inc. Glen Burnie, MA) and following the manufacturer’s instructions. Using this in vivo delivery method, it has been demonstrated that integration of the naked siRNA into neural cells is effective as early as 3 hours after injection and persists up to 7 days.

Collection of Tissue Samples from RVLM
At the age of 3, 6, 12 or 16 weeks, or at various time intervals after treatment, SHR or WKY rats were killed with an overdose of pentobarbital sodium (100 mg/kg, IP) and perfused intracardially with warm saline. The brain was rapidly removed and immediately frozen on dry ice. Medulla oblongata covering RVLM was blocked between 0.5 and 1.5 mm rostral to the obex, which was adopted from the atlas of Watson and Paxinos17 and served as the anatomical landmark. Both sides of the ventrolateral medulla covering RVLM (approximately 1.5- to 2.5-mm lateral to the midline and medial to the spinal trigeminal tract) were collected by micropunches with a 1-mm inner diameter burr.1,2 Medullary tissues collected from the same experimental groups were pooled and stored at -80°C prior to protein analysis.

Protein Extraction
Total protein from RVLM was extracted with ice-cold lysis buffer. Protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin and 20 μg/ml phenylmethylsulfonyl fluoride)
and phosphatase inhibitors (2 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate) were included in the lysis buffer to prevent protein degradation. Solubilized proteins were centrifuged at 20000 g at 4°C for 15 minutes, and proteins in the supernatant were quantified by the Bradford assay with a protein assay kit (Bio-Rad, Hercules, CA).

**Western Blot Analysis**

Proteins (10 to 50 µg) were separated using 7.5% SDS-PAGE and transferred to PVDF membrane for 1.5 hours at 4°C, using a Bio-Rad miniprotein-III wet transfer unit. The transfer membranes were then incubated with blocking solution (5% nonfat dried milk dissolved in Tris-buffered saline–Tween buffer (pH 7.6, 10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) for 1 hour at room temperature. The primary antiserum used included rabbit polyclonal or mouse monoclonal antiserum against GRP78 (1:1000; BD Biosciences), phosphorylated-eIF2α (p-eIF2α, 1:1000; Abcam, Cambridge, UK), eIF2α (1:2000; Abcam), LC3 I and II (1:1000; Cell signaling, Danvers, MA), LAMP-2 (Invitrogen, Carlsbad, CA), caspase 3 (1:1000, Cell signaling), caspase 12 (1:1000, Cell signaling), and α-tubulin (1:5000; Calbiochem, Darmstadt, Germany). The secondary antiserum used included horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA). Specific antibody-antigen complex was detected using an enhanced chemiluminescence Western Blot detection system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The amount of detected protein was quantified by the Photo-Print Plus software (ETS Vilber-Lourmat, Marne-la-Vallée, France), and was expressed as the ratio to α-tubulin or total eIF2α protein.

**Phos-tag Gels**

Phos-tag gel was carried out with minor modifications of procedures previously described. In brief, Phos-tag gel was prepared in the same way as in regular SDS-PAGE gels, except that 50 µM Phos-tag (AAL-107; NARD institute, Tokyo, Japan) and 50 µM MnCl₂ were included. The gel was run at 50 V for 30 minutes followed by 100 V for 3 hours. This was followed by soaking the gels in an ice-cold transfer buffer containing 1 mM EDTA for 10 minutes before transferring onto a PVDF membrane. The eIF2α antibody (1:2000; Abcam) was applied, and p-eIF2α was detected at 40 kDa versus un-phosphorylated eIF2α (u-eIF2α) at 34 kDa. The eIF2α blots were re-probed with HSP60 (60 kDa; Santa Cruz) as a position/loading control. To ensure that band shift in the Phos-tag gel is due to phosphorylation of eIF2α, 25 µg total protein of RVLM was incubated with 1µl of a wide range phosphatase inhibitor cocktail (cat. 95726; Sigma-Aldrich) in 1X alkaline phosphatase buffer at 37°C for 1 hour. Reaction was stopped by adding 2X SDS sample buffer and incubated at 100°C for 10 minutes. Data were expressed as a percentage of p-eIF2α or u-eIF2α relative to total eIF2α (p-eIF2α + u-eIF2α) expression.

**Measurement of Reactive Oxygen Species**

For measurement of the reactive oxygen species (ROS) in RVLM, extracted proteins were reacted with the oxidation-sensitive fluorescent probe dihydroethidium (DHE, 1 µM; Invitrogen). RVLM tissue was homogenized in a 20 mM sodium phosphate buffer (pH 7.4) that contains 0.01 mM EDTA by a glass-to-glass homogenizer. The homogenate was subjected to low speed centrifugation at 1000 g for 10 minutes at 4°C to remove nuclei and unbroken cell debris. The pellet was discarded and the supernatant was obtained immediately for ROS measurement. The suspension was reacted with the oxidation-sensitive fluorescent probe DHE at 37°C for 15 minutes under protection from light. The fluorescence was analyzed in a microplate reader (FluorStar; Biodirect, Inc., Taunton, MA).

**Isolation of RNA and Reverse Transcription Real-Time Polymerase Chain Reaction**

Total RNA from RVLM was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA).
according to the manufacturer’s protocol. All RNA isolated was quantified by spectrophotometry and the optical density 260/280 nm ratio was determined. Only RNA samples with a ratio higher than 1.8 were used for subsequent analysis. Reverse transcription (RT) reaction was performed using a RevertAid™ First Strand cDNA Synthesis Kit (Ferments Inc.) for the first strand cDNA synthesis. Real-time polymerase chain reaction (PCR) analysis was performed by amplification of cDNA using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The primers used in real-time PCR amplification were designed using Roche LightCycler probe design software 2.0 based on sequence information from the NCBI database, and were synthesized by Genomics BioSci & Tech (Taiwan). PCR reaction for each sample was carried out in triplicate for all the cDNA and for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. The primer pairs for amplification of were: GRP78 cDNA22: 5’-TACTGGACTCAGGAACTTTCGT-3’ (forward), 5’-ACAAACATTATTTGTGCTCTTTGGTA-3’ (reverse); GAPDH cDNA were: 5’-TCCATGACAACTTTGGCATG-3’ (forward), 5’-TCACGCCACAGCTTTCCAG-3’ (reverse). The amplification protocol for cDNA was a 10-minute denaturation step at 95°C for polymerase activation, followed by 40 cycles consisting of 15 seconds at 95°C, 15 seconds at 60°C, and 60 seconds at 72°C. After slow heating (0.1°C per second) the amplified product from 65°C to 95°C to generate a melting temperature curve, which serves as a specificity control, the PCR samples were cooled to 40°C. The PCR products were subsequently subjected to agarose gel electrophoresis for further confirmation of amplification specificity. Fluorescence signals from the amplified products were quantitatively assessed using the LightCycler® software program (version 3.5). Second derivative maximum mode was chosen with baseline adjustment set in the arithmetic mode. The relative change in Grp78 mRNA expression was determined by the fold-change analysis.

**Immunohistochemistry**

Animals were perfused transcardially with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) under deep pentobarbital anesthesia (100 mg/kg, IP), and the brain stem was removed and post-fixed overnight in the same fixative, followed by 30% sucrose solution for at least 3 days. 35-μm coronal sections of the medulla oblongata were cut using a cryostat (Leica, Houston, TX). The sections were rinsed for 30 minutes in PBS. After pre-absorption in gelatin (0.375%), normal horse serum (3%) and triton-X 100 (0.2%) in PBS, the sections were incubated with a mouse monoclonal antibody against GRP78 (1:500; Santa-Cruz Biotechnology) or a rabbit polyclonal antibody against phospho-eIF2α (1:500; Abcam, Cambridge, UK) at room temperature overnight and then rinsed 3 times in PBS. After incubation in biotinylated horse anti-mouse IgG or anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) for 1 hour, the sections were rinsed 3 times in PBS and incubated with the AB complexes for 30 minutes (Vectastain ABC elite kit, Vector Laboratories, Burlingame, CA). This was followed by washing the sections 2 times in PBS and incubated with DAB substrate kit for 10 minutes (Vector Laboratories). Sections were mounted and observed under a light microscope (Olympus Optical, Tokyo, Japan).

For quantification of immunoreactive cells in RVL, the medullary sections that cover the caudal-to-rostral axis of RVL were collected at 105-μm intervals. The GRP78 or p-eIF2α immunoreactive cells on both sides of RVL from all collected sections were counted by two independent individuals in a single-blind fashion. Total number of counted cells was divided by the number of sections to represent the average number of immunoreactive cells on both sides of RVL per section. A total of 3 animals were used for quantification of the immunohistochemical data.

**Double immunofluorescence staining and laser confocal microscopy**

The procedures for double immunofluorescence staining were similar to those reported previously.2,23 In brief, free-floating 20-μm sections of the medulla oblongata containing RVL were incubated with a rabbit polyclonal antiserum against GRP78 (1:500; Santa-Cruz Biotechnology) or phospho-eIF2α (1:500; Abcam, Cambridge, UK), together with a mouse monoclonal antiserum against a specific neuron marker,
neuron-specific nuclear protein (NeuN) (1:1000; Chemicon, Temecula, CA), an astrocyte marker, GFAP (1:1000; Dako, Denmark) or a microglia marker, Iba-1 (1:1000; Wako, Japan). The sections were subsequently incubated concurrently with a goat anti-rabbit IgG conjugated with Alexa Fluor 488 for GRP78 or phospho-eIF2α, or a goat anti-mouse IgG conjugated with Alexa Fluor 568 for NeuN, GFAP or Iba-1. Viewed under a Fluorview FV10i laser scanning confocal microscope (Olympus), immunoreactivity for GRP78 or phospho-eIF2α exhibited green fluorescence and NeuN, GFAP or Iba-1 manifested red fluorescence. The co-localization of red and green fluorescence on merged images indicated double labeling.

**Histology**

With the exception of animals used for biochemical analyses and immunohistochemical staining, the brain stem was removed from animals after they were killed by an overdose of sodium pentobarbital (100 mg/kg, IV), and fixed in 30% sucrose in 10% formaldehyde-saline solution for ≥ 72 hours. Frozen 25-μm sections of the medulla oblongata were stained with 1% Neural Red for histological verification of the location of microinjection sites.

**Statistical Analysis**

All values are expressed as means ± SEM. One-way or 2-way analysis of variance with repeated measures was used to assess group means, as appropriate, to be followed by the Scheffé multiple-range test for post hoc assessment of individual means. In some cases, Student’s t-test was used. P < 0.05 was considered statistically significant.
References


**Figure S1.** Representative SDS-PAGE (for GRP78) or Phos-tag (for eIF2α) gels (inset) or densitometric analysis of protein level of phosphorylated or unphosphorylated eIF2α (p-eIF2α or u-eIF2α), GRP78 or α-tubulin in RVLM (A) or the ventromedial medulla (B) of 16-week-old SHR or the age-matched WKY rats. Note that as shown in the representative Phos-tag gels in (A), addition of a phosphatase inhibitor cocktail (PI) that covers a wide range of phosphatases blocks the band shift due to eIF2α phosphorylation. Values are mean ± SEM of quadruplicate analyses on samples pooled from 4 to 8 animals in each group. Data in eIF2α are expressed as a percentage of p-eIF2α relative to total eIF2α (t-eIF2α). *P < 0.05 versus age-matched WKY rat group in the Student’s t-test.
Figure S2. Expression of GRP78 mRNA detected from RVLM in SHR (3-, 6-, 12- or 16-week-old) or age-matched WKY rats. Values are mean ± SEM of quadruplicate analyses on samples pooled from 6 to 8 animals in each group. *P < 0.05 versus age-matched WKY rats in the post hoc Scheffé multiple range analysis.
Figure S3. Changes in basal mean arterial pressure (MAP) (A) and representative gels (inset) or densitometric analysis of protein level of GRP78, p-\(\text{eIF}2\alpha\) or \(\text{eIF}2\alpha\) (B) detected from RVLM on day 7 following oral intake of captopril (100 mg·kg\(^{-1}\)·day\(^{-1}\)) or amlodipine (5 mg·kg\(^{-1}\)·day\(^{-1}\)) in SHR (16-week-old) or age-matched WKY rats for 7 days. Values are mean ± SEM of 5-8 animals in each group (A) or quadruplicate analyses on samples pooled from 5 to 6 animals in each group (B). *\(P < 0.05\) versus saline group of SHR (A) or WKY rats (B) in the post hoc Scheffé multiple range analysis.
Figure S4. Temporal changes in MAP (A) or power density of the low-frequency (LF) component of systolic blood pressure spectrum (B) after intracisternal infusion of salubrinal (12 pmol•kg\(^{-1}\)•day\(^{-1}\)) or 2% DMSO in WKY rats. Values are mean ± SEM of 6-8 animals in each group. No significant difference between groups (\(P > 0.05\)) in one-way ANOVA.
Figure S5. Changes in tissue level of ROS in RVLM of SHR detected on day 7 after intracisternal infusion tempol (10 or 100 pmol•kg\(^{-1}\)•day\(^{-1}\)) (A) or salubrinal (3 or 12 pmol•kg\(^{-1}\)•day\(^{-1}\)) (C), or temporal changes in MAP of SHR following intracisternal infusion of tempol (10 or 100 pmol•kg\(^{-1}\)•day\(^{-1}\)) (B). Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group (A,C) or 6-8 animals in each group (B). *P < 0.05 versus age-matched WKY group, and #P < 0.05 versus control SHR group in the post hoc Scheffé multiple range analysis.
**Figure S6.** Representative gels (inset) or densitometric analysis of protein level of GRP78 (A) and total or phosphorylated eIF2α (p-eIF2α) (B) detected from RVLM of WKY rats on day 7 after intracisternal infusion of aCSF or Ang II (500 pmol•kg⁻¹•day⁻¹) alone or with additional microinjection bilaterally into RVLM of tempol (100 pmol) on day 7 after Ang II infusion. Values are mean ± SEM of quadruplicate analyses on samples pooled from 6 to 8 animals in each group. *P < 0.05 versus control group, and #P < 0.05 versus Ang II group in the post hoc Scheffé multiple range analysis.
Figure S7. Representative gels (inset) or densitometric analysis of protein level of GRP78 or ratio between LC3-II and LC3-I protein level detected from RVLM of SHR (16-week-old) on day 3 after microinjection bilaterally into RVLM of small interfering RNA against GRP78 (siGRP78) or LC3-II (siLC3-II) or its control (siControl). Values are mean ± SEM of quadruplicate analyses on samples pooled from 6 to 7 animals in each group. *P < 0.05 versus siControl group in the post hoc Scheffé multiple range analysis.
**Figure S8.** Tissue level of ROS detected from RVLM of SHR 90 minutes after microinjection bilaterally into RVLM of 3-MA (5 nmol), bafilomycin A1 (BAF A1, 100 nmole), or on day 3 after microinjection bilaterally into RVLM of siControl, siGRP78 or siLC3-II. Baseline values from WKY rats are included for comparison. Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. *P < 0.05 versus WKY group in the post hoc Scheffé multiple range analysis.
Figure S9. Representative Phos-tag gels (inset) or densitometric analysis of protein level of phosphorylated or unphosphorylated elF2α (p-elF2α or u-elF2α) in RVLM detected on day 3 after microinjection of siGRP78 or siControl into the bilateral RVLM of SHR (16-week-old). The expression of elF2α in RVLM of the age-matched WKY rats is included for comparison. Values are mean ± SEM of quadruplicate analyses on samples pooled from 5 to 6 animals in each group. Data in elF2α are expressed as a percentage of p-elF2α relative to total elF2α (t-elF2α). *P < 0.05 versus age-matched WKY group in the post hoc Scheffé multiple range analysis.