**ERp44 Exerts Redox-Dependent Control of Blood Pressure at the ER**

**Highlights**
- Genetic loss of ERp44 destabilizes angiotensin II and causes hypotension in mice
- ERp44 binds to ERAP1 in the ER in a redox-dependent manner
- ERp44 suppresses the release of ERAP1, which cleaves angiotensin II in plasma
- ERp44-ERAP1 complex inhibits hypotension during systemic inflammation

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**In Brief**
Hisatsune et al. show that ERp44 inhibits hypotension during inflammation by regulating angiotensin II. ERp44 does this by sequestering the angiotensin II peptidase, ERAP1, via a redox-regulated disulfide bond with ERAP1.
**ERp44 Exerts Redox-Dependent Control of Blood Pressure at the ER**

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**SUMMARY**

Blood pressure maintenance is vital for systemic homeostasis, and angiotensin II is a critical regulator. The upstream mechanisms that regulate angiotensin II are not completely understood. Here, we show that angiotensin II is regulated by ERp44, a factor involved in disulfide bond formation in the ER. In mice, genetic loss of ERp44 destabilizes angiotensin II and causes hypotension. We show that ERp44 forms a mixed disulfide bond with ERAP1, an aminopeptidase that cleaves angiotensin II. ERp44 controls the release of ERAP1 in a redox-dependent manner to control blood pressure. Additionally, we found that systemic inflammation triggers ERAP1 retention in the ER to inhibit hypotension. These findings suggest that the ER redox state calibrates serum angiotensin II levels via regulation of the ERp44-ERAP1 complex. Our results reveal a link between ER function and normotension and implicate the ER redox state as a potential risk factor in the development of cardiovascular disease.

**INTRODUCTION**

Blood pressure regulation is a vital component of homeostasis. Therefore, changes in blood pressure are an important indicator of disease. Normotension is maintained by several mechanisms, most notably, the level of angiotensin in the circulatory system, which depends on an intricate balance between angiotensin synthesis in the renin-angiotensin system (RAS) and its metabolism during circulation (de Gasparo et al., 2000; Johnston, 1990). However, the cellular mechanisms of blood pressure regulation upstream of angiotensin II turnover are poorly understood.

Angiotensin II is produced when angiotensinogen is cleaved by renin to form the precursor angiotensin I that is further trimmed by angiotensin-converting enzyme (ACE) to angiotensin II, which has diverse biological functions, including arterial blood vessel constriction. Aminopeptidases and ACE2 metabolize angiotensin II during circulation (Ohishi et al., 2013; Shi et al., 2010; Clarke and Turner, 2012). These mechanisms stringently regulate circulating plasma angiotensin II to maintain blood pressure and fluid homeostasis. However, pathological conditions like inflammation and metabolic disease, which produce stress on various organs at the cellular level, can disrupt angiotensin regulation, leading to an unfavorable rise or drop blood pressure and subsequent cardiovascular and renal malfunction.

The ER houses the machinery for folding and assembly of a vast number of secretory and membrane proteins and is tasked with quality control prior to secretion (Elligaard and Helenius, 2003; Ellgaard and Ruddock, 2005). The ER is particularly sensitive to cellular stress, such as that caused by chronic inflammatory or degenerative diseases (Hotamisligil, 2010), and can alter the internal redox state of the ER affecting protein folding and complex formation. The stability and function of proteins and protein complexes often depends on the formation of inter- and intramolecular disulfide bonds, a process mediated by ER enzymes under oxidizing conditions (Amfinsen, 1973).

Disulfide bonds help to define protein structure. Their formation is regulated by oxidoreductases and chaperones including Erp1, protein disulfide isomerase (PDI), and PDI-like oxidoreductases (Ellgaard and Ruddock, 2005; Hatahet and Ruddock, 2009; Margittai and Sitia, 2011; Sevier and Kaiser, 2008). ERp44, an ER resident thioredoxin (TRX)-like motif-containing protein, also contributes to disulfide bond formation, and cell culture studies suggest that it plays a crucial role in the thiol-mediated retention and maturation of several secretory proteins within the ER (Anelli et al., 2002, 2007; Kakihana et al., 2013; Long et al., 2010; Phillips et al., 2009; Wang et al., 2007). ERp44 also controls Ca²⁺ signaling from the ER via the type 1 inositol 1,4,5-trisphosphate receptor, in HeLa cells (Higo et al., 2005). A recent study demonstrated the importance of ERp44 in embryonic heart development, but its physiological role in adult tissues in vivo remains unknown (Wang et al., 2014).

In this study, we show that ERp44 exerts blood pressure control by regulating serum angiotensin levels. Genetic loss of ERp44 in mice destabilizes angiotensin II in the bloodstream and causes profound hypotension. We identified the aminopeptidase ERAP1 as a binding partner of ERp44 in the ER and demonstrate that ERp44 redox dependently controls the intracellular localization of ERAP1, which digests plasma...
angiotensin II when released into the bloodstream. We also show that during systemic inflammation, the thiol-based ERp44-ERAP1 association is increased to counteract sepsis-induced hypotension. Thus, our findings establish a blood pressure regulatory mechanism by the ERp44-ERAP1 complex that is dependent on the redox state of the ER.

RESULTS

Erp44–/– Mice Have Hypotension and Exhibit Altered Kidney Morphology

To examine the effect of ERp44 loss in vivo, we generated ERp44-knockout (Erp44–/–) mice on the C57BL/6 genetic background by homologous recombination (Figures S1A–S1C). ERp44 expression was absent from tissues of C57BL/6 Erp44–/– mice at birth, confirming the deletion of ERp44 expression (Figure S1D).

Erp44–/– mice could suckle and breathe, but most Erp44–/– mice died within 24 hr after birth, with only a few (~0.15% [4/2,634 pups]) reaching adulthood. However, most F2 Erp44–/– mice from a mixed genetic background (FVB/NJ × C57BL/6; Figure S1E) grew to adulthood, although their body size was smaller than control mice, similar to the C57BL/6 genetic background strain (Figure S1F). The birth rates of Erp44–/– mice were 12% (45/382) for C57BL/6 and 14% (21/152) for FVB/NJ × C57BL/6 genetic backgrounds, respectively, compared to the expected Mendelian birth rate of 25%. On the contrary, survival to adulthood was very different for pure C57BL/6 (0.15% [4/2,634]) and mixed FVB/NJ × C57BL/6 genetic backgrounds, perhaps due to genetic factors.

C57BL/6 Erp44–/– pups had decreased urine volume at birth (Figure 1A), and histological analysis of the kidneys demonstrated distension of cortical distal tubules (Figure 1B, asterisks) and a mild hypoplasia of the renal papilla in Erp44–/– mice. Such alteration was never observed in Erp44+/+ or Erp44+/– mice. Since decreased urine volume and distension of distal tubules were also observed in FVB/NJ × C57BL/6 mixed genetic Erp44–/– mice at birth (data not shown), these phenotypes...
seemed not to be directly related to the early postnatal death of C57BL/6 Erp44+/− mice. Kidneys of the surviving adult C57BL/6 Erp44+/− mice as well as those of FVB/NJ × C57BL/6 Erp44+/− mice appeared histologically normal except for a disproportional reduction of the cortex and the outer medulla (Figures S1G and S1H). In these Erp44+/− animals, renal glomeruli were slightly diminished in size, and proximal and distal tubules were strikingly shortened as compared with wild-type (WT) siblings. There was no apparent abnormality in Erp44+/− kidney morphology in either C57BL/6 or FVB/NJ × C57BL/6 mixed genetic background (data not shown; n ≥ 3 for each genotype were evaluated).

The abnormalities in Erp44+/− mice resemble those of angiotensinogen-deficient mice (Nimura et al., 1995). Thus, we hypothesized that Erp44+/− mice had abnormalities in the renin-angiotensin system controlling blood pressure. We measured blood pressure in adult Erp44+/− mice and found a blood pressure reduction in both male and female Erp44+/− mice compared with control Erp44+/+ or Erp44+/−/C0 mice on FVB/NJ × C57BL/6 genetic background (Figure 1C). The three surviving adult C57BL/6 male Erp44+/− mice also showed a reduction of blood pressure as compared to Erp44+/−/C0 littermates (Figure S1I). Because no WT littermates were available for comparison in this case, Erp44+/−/C0 mice were used as controls.

Decreased Angiotensin Stability in ERp44-Deficient Plasma

We first examined the mRNA expression levels of angiotensinogen, renin, and ACE1, the essential substrates and enzymes, to see if angiotensin II production was abnormal in Erp44+/−/C0 mice. However, expression levels of these genes were not decreased in FVB/NJ × C57BL/6 Erp44+/−/C0 mice as compared to controls (Figure S1J).

Next, we quantified the concentrations of angiotensin II, III, and IV in mouse plasma by mass spectrometry, using stable-isotope-labeled angiotensin II1–18 as the internal standards, for FVB/NJ × C57BL/6 genetic background Erp44+/−/C0 mice. As shown in Figure 1D, we detected single peaks for angiotensin II, III, and IV on mass chromatograms with retention times at 12.07, 11.22, and 12.64 min, respectively. We quantified the absolute concentration of angiotensin II and found that adult Erp44+/−/C0 mice had a decreased angiotensin II concentration as compared to the control littermate Erp44+/+ mice (Figure 1E). The angiotensin III and IV concentrations in Erp44+/−/C0 mice were also slightly decreased, but not significantly different, compared with those in Erp44+/+ and Erp44+/−/C0 mice. The ratio of angiotensin III/IV to II concentration was elevated in Erp44+/−/C0 plasma as compared to that in control (Erp44+/+ and Erp44+/−/C0) mouse plasma (Figure 1F).

We hypothesized that angiotensin II may be less stable in Erp44+/−/C0 mouse plasma. We treated synthetic angiotensin II (Peptide Institute) with mouse plasma for various durations (10 min, 30 min, and 60 min) in vitro and compared the degradation rates in plasma between control and Erp44+/−/C0 FVB/NJ × C57BL6 mice by high-performance liquid chromatography (HPLC). The angiotensin II peak decreased and an additional three peaks appeared in a time-dependent manner (Figures 2A and S2). Using MALDI-TOF tandem mass spectrometry (MS/MS) analysis, we identified the three peaks as angiotensin III, Tyr-Ile-His-Pro-Phe, and angiotensin IV, respectively (data not shown). We also examined the time-dependent degradation of angiotensin II and production of angiotensin III, IV, and YHFP peptide in the assay using plasma from FVB/NJ × C57BL6 Erp44+/−/C0 mice and their littermate controls and found a more rapid digestion of angiotensin II and production of YHFP peptide in Erp44+/−/C0 plasma compared with Erp44+/+ or Erp44+/−/C0 plasma (Figure 2B). We concluded that angiotensin II and its metabolites, angiotensin III and IV, are less stable in Erp44+/−/C0 plasma than in control littermate plasma from Erp44+/+ and Erp44+/−/C0 mice and were more rapidly metabolized to the YHFP peptide (Figure 2B).

**Erp44+/+ Mice Exhibit Significantly Elevated Leucine Aminopeptidase Activity in Plasma**

ERp44 plays an important role in tethering various molecules lacking ER retention signals (e.g., adiponectin, immunoglobulin M [IgM]) to the ER to regulate their maturation and secretion (Anelli et al., 2003, 2007; Long et al., 2010; Phillips et al., 2009; Wang et al., 2007). Thus, we hypothesized that ERp44 interacts with an enzyme that digests angiotensin II in mouse plasma. We found that the activity of leucine aminopeptidase (LAP) was greatly increased in Erp44+/−/C0 mouse serum, whereas there were no changes in serum glutamic oxaloacetic transaminase (GOT) activity, glucose, albumin, uric acid (UA), or urea nitrogen (BUN) (Figure 2C).

**Identification of ERAP1 as an ERp44-Interacting Protein**

To identify ERp44-interacting proteins, we immunoprecipitated ERp44 from mouse liver lysates, which contain a high level of ERp44, analyzed the co-immunoprecipitated proteins by silver staining (Figure 3A), and found a high-molecular-weight protein band that was only observed in the presence of N-ethyl maleimide (NEM), which inhibits intermolecular thiol-disulfide exchange (Figure 3A, asterisk), suggesting a complex whose interaction is redox dependent and requires a disulfide bond. We analyzed the band by MS/MS and identified the ERp44-interacting protein as ER aminopeptidase 1 (ERAP1), a member of the M1 family of zinc metalloproteases characterized by a catalytic HEXXH(X)12E Zn-binding motif (Tsujimoto and Hattori, 2005), also known as ER aminopeptidase associated with antigen processing (ERAAP; Serwold et al., 2002), adipocyte-derived leucine aminopeptidase (A-LAP; Hattori et al., 1999), puromycin-insensitive leucine-specific aminopeptidase (PILS-AP; Schomburg et al., 2000), and aminopeptidase regulator of tumor necrosis factor receptor type I shedding (ARTS-1; Cui et al., 2002) (Figure S3). Since polymorphisms are associated with essential hypertension (Yamamoto et al., 2002) and ERAP1 cleaves angiotensin II in vitro (Hattori et al., 2000), we analyzed ERAP1 as a possible explanation for hypotension in Erp44+/−/C0 mice.

Overexpression of ERAP1 and ERp44 in HeLa cells showed the formation of the ERp44-ERAP1 complex in the presence of NEM (Figures 3B and 3D). In addition, DTT treatment to inhibit disulfide bond formation decreased the amount of ERAP1 co-immunoprecipitated with ERp44 by 60% (Figures 3C and 3D). Furthermore, the putative ERp44-ERAP1 complex band migrated at >250 kDa on SDS-PAGE under non-reducing conditions.
conditions, but not under reducing conditions (Figure 3F). These results suggested that ERp44 binds to ERAP1 in a redox-dependent manner.

**C29 of ERp44 Forms a Mixed Disulfide Bond with C487 of ERAP1**

To determine the binding sites between ERp44 and ERAP1, we mutated two cysteines (C29 and C63) of ERp44 to serines, because these cysteine residues can form intermolecular disulfide bonds within the TRX-like domain and particularly because C29 is the CXXS cysteine at the amino-terminus of the active-site helix, which is expected to be involved in interaction with target proteins (Anelli et al., 2007). We found that ERp44 C29S had greatly decreased affinity for ERAP1 binding (Figures 4A and 4B), although a weak degree of binding was still observed. The residual binding was not dependent on a disulfide bond, because it was not detected on SDS-PAGE under non-reducing conditions (Figure S4A). ERp44 C63S also had a decreased affinity for ERAP1, but a covalent complex still formed between the two proteins (Figure S4A). We therefore constructed several deletion mutants of ERp44 and found that the residual binding was due to a weak physical affinity of the b + b′ domain of ERp44 to ERAP1 (Figure 4C). Together, these results suggested that C29 of ERp44 is a major binding site for ERAP1.

We also aligned the ERAP1 amino acid sequence with placental leucine aminopeptidase (P-LAP), which belongs to the same M1 family but has a transmembrane domain with a distinct intracellular localization at the plasma membrane (Rogi et al., 1996), from various species. ERAP1 contains an additional amino acid sequence (aa 475–501) within the extracellular domain compared with P-LAP (Figure 4D), suggesting this region might be essential for ERAP1 retention within the ER (Hattori et al., 2012). We identified two cysteine residues (C475 and C487 in mouse and C486 and C498 in human ERAP1, respectively), mutated these residues to alanine, and found that mutation of C487A, but not C475A, significantly decreased the ERp44-ERAP1 association (Figures 4E and 4F). These results suggest that ERp44 binds to ERAP1 via a disulfide bond between C29 of ERp44 and C487 of ERAP1.
ERp44 Contributes to Retention of ERAP1 within the ER

ERp44 and ERAP1 overexpressed in HeLa cells extensively co-localized with protein disulfide isomerase (PDI), a marker of ER, and also partially co-localized with GM130, a Golgi marker (Figure S4B), and overexpressed ERAP1 and ERp44 colocalized in HeLa cells (Figure 4G). Endogenous ERAP1 was detectable by immunocytochemistry in ERp44-overexpressing HeLa cells (arrows), but not in non-overexpressing cells (asterisks in Figure 4H). Since ERAP1 has no ER retention signal itself and is known to be secreted from the ER when exogenously overexpressed in COS cells (Hattori et al., 1999), these results suggested that ERAP1 accumulated in the ER of the ERp44-transfected cells via ERp44-mediated retention and that ERp44 expression level affected ERAP1 content within the ER.

To confirm ERp44-mediated retention of ERAP1 within the ER, we measured LAP activity in the culture medium of HeLa cells overexpressing WT and mutant forms of ERp44 and ERAP1 (Figure 4I). We detected strong LAP activity in the culture medium of HeLa cells overexpressing WT ERAP1. Since almost no LAP activity was observed in the culture medium of HeLa cells without interferon γ (IFN-γ) treatment as compared to DMEM, the increased LAP activity was derived from the activity of the transfected ERAP1. Co-expression of WT ERp44 significantly decreased ERAP1 activity in the culture medium; however, co-expression of C29S ERp44 or the ERp44 mutant lacking the ER retention signal, RDEL (ERp44 delta) (Figure 4I), had a negligible effect. We also found that ERAP1 activity in the medium of HeLa cells expressing the C487A ERAP1 mutant and WT ERp44 was elevated compared to cells expressing WT ERAP1 (Figure 4J). The non-covalent weak physical interaction of ERAP1 with ERp44 (Figure 4C) may contribute to the decreased LAP activity in the culture medium of cells expressing C29S ERp44 or C487A ERAP1 mutants as compared to those expressing ERAP1 alone in Figures 4I and 4J.
To confirm that ERp44 regulates retention of ERAP1, we examined ERAP1 localization in C57BL/6 background Erp44+/+ and Erp44−/− mouse embryonic fibroblasts (MEFs) that were treated with IFN-γ for ERAP1 induction (Serwold et al., 2002). A significant amount of ERAP1 protein was observed in the P2/P3 membrane fraction of Erp44+/+, but not Erp44−/−, cells (Figures 5A and 5B), suggesting that the ERp44 expression level controls the amount of the ER-resident ERAP1. We confirmed this finding using small interfering RNA (siRNA) knockdown of ERp44 in HeLa cells (Figures S5A and S5B). ERAP1 expression was strongly detected in Erp44−/− MEF lysates but was very faint in Erp44+/− cell lysates (Figure 5A). Conversely, the ERAP1 protein level in the culture medium was significantly elevated in Erp44−/− MEF, but not in Erp44+/+ MEF, cells (Figure 5B). The LAP activity in the culture medium of Erp44−/− MEF cells was increased compared to WT MEF cells (Figure 5C), and this increase was not observed when we overexpressed red fluorescent protein (RFP)-tagged ERp44, but not RFP, in the...
Erp44−/− MEF cells (Figures 5D and S5). In addition, pre-treatment of the Erp44−/− MEF culture medium with the anti-ERAP1 antibody, but not the immunoglobulin G (IgG)-conjugated affinity beads, prevented the increase in LAP activity (Figure 5E). ERAP1 mRNA expression levels were similar between Erp44+/+ and Erp44−/− MEF cells (Figure 5F), supporting the conclusion that secretion of ERAP1 depends on the presence of Erp44.

We additionally examined ERAP1 protein levels in plasma of FVB/NJ × C57BL/6 background Erp44−/−, Erp44+/+, and Erp44−/− mice and found an elevation of ERAP1 in the Erp44−/− mouse plasma as compared to Erp44+/+ or Erp44−/− by immunoblotting (Figures 5G and 5H). Furthermore, the ERAP1-ERp44 complex as well as the ERAP1 band was undetectable in Erp44−/− liver lysates on non-reducing SDS-PAGE, which was likely due to ERAP1 secretion into the bloodstream (Figure S5D),
since the expression levels of ERAP1 mRNA in liver and kidney were not different among Erp44+/−, Erp44+/−, and Erp44−/− mice (Figure S5E).

The molecular weight of ERAP1 in the Erp44−/− mouse plasma was slightly larger than that of ERAP1 in the cell lysates of Erp44+/− MEF cells, and deglycosidase treatment abolished the difference (Figure 5I), suggesting that the secreted ERAP1 in the plasma of Erp44−/− mice was more glycosylated than those in Erp44+/− mice while traveling through the ER-Golgi network.

To directly demonstrate the release of ERAP1 from Erp44−/− cells, we examined ERAP1 dynamics in Erp44−/− and Erp44+/− MEF cells by pulse labeling. The cells were pulse labeled with 35S Met and 35S Cys for 30 min, and labeled ERAP1 was immunoprecipitated from the cell lysates or culture medium after 0, 1, 3, and 5 hr. As shown in Figure 5J, doublet ERAP1 bands were detected in Erp44−/− cell lysates after 30 min of pulse labeling. The lower band was a non-glycosylated form and rapidly disappeared within 1 hr. The intensity of the upper ERAP1 band was relatively constant in Erp44+/− cell lysates for at least 5 hr. By contrast, in Erp44−/−/− cell lysates, the ERAP1 band gradually disappeared (Figure 5J, right). In addition, a third, slow-moving band appeared within 1 hr and remained constant at 3 and 5 hr. Instead, a band with the same molecular weight as the upper-most band appeared in the culture medium within 1 hr and increased in a time-dependent manner (Figure 5J, lower panel). Thus, the data strongly suggested that ERAP1 produced by Erp44−/− cells was released into the medium in a highly glycosylated form.

Immunodepletion of ERAP1 from Erp44−/− Mouse Plasma Increased Angiotensin II Stability

To examine whether the difference in angiotensin II stability in Erp44−/− mouse plasma was indeed due to elevated plasma ERAP1 levels, we depleted ERAP1 from mouse plasma with anti-ERAP1 antibody and performed the angiotensin II digestion assay. Immunodepletion of ERAP1 with anti-ERAP1 antibody, but not IgG, negated the elevated LAP activity of the Erp44−/− mouse plasma to the level seen in controls, indicating that the elevated LAP activity in plasma of Erp44−/− mice was largely due to ERAP1 (Figure 5K). In addition, angiotensin II instability diminished when ERAP1 was pre-absorbed from the plasma with anti-ERAP1 antibodies, but not IgG (Figure 5L). These results suggested that deletion of ERp44 induced hypotension in mice due to decreased angiotensin II stability caused by an elevation of ERAP1 in plasma.

ERp44 Counteracts Acute Hypotension by Retaining ERAP1 within the ER during Systemic Inflammation

Sepsis is a fatal clinical syndrome characterized by hypotension and a systemic inflammatory response and is reported to cause ER stress (Zhang et al., 2006). The hypotension observed during sepsis is a result of several factors, not all of which are clearly understood. We hypothesized that there is a physiological role of the ERp44-ERAP1 interaction in blood pressure control during sepsis. Therefore, we measured blood pressure in Erp44+/+ and Erp44−/− mice upon sepsis. Erp44+/+ mice express half the amount of ERp44 (Figure S6A) and demonstrated a much greater drop in blood pressure than the Erp44+/+ mice upon sepsis (Figures 6A and 6B), suggesting that a decrease in the amount of ERp44 in Erp44+/− mice hindered their ability to regulate blood pressure in response to a systemic stress.

To examine whether ERp44 and ERAP1 are part of the body’s response to systemic inflammation, we examined the expression of these genes and found that ERp44 and ERAP1 expression increased in the liver of Erp44+/− mice, in addition to the expression of molecular chaperones, such as BIP and PDI, that are known to be induced by sepsis (Zhang et al., 2006) (Figures 6C, 6D, and S6B).

We additionally found an increase in ERp44-ERAP1 complex formation in the liver, spleen, and kidney of Erp44+/− mice upon sepsis by co-immunoprecipitation and on non-reducing SDS-PAGE (Figures 6E, 6F, and S6C–S6E).

LAP activity was more elevated in the plasma of Erp44+/− mice than in the Erp44−/− mice upon sepsis. In contrast, no difference in the GOT activity was observed between Erp44+/− and Erp44−/− mice (Figure 6G). These results suggested that ERp44 prevents hypotension by retaining ERAP1 within the ER during systemic inflammation.

Erp44+/− mutants have a lower blood pressure compared to Erp44+/+ and Erp44−/− mice under healthy conditions. Therefore, we did not include them in this experiment, as any changes in blood pressure upon sepsis could not be directly compared to Erp44+/+ and Erp44−/− mice and the lack of ERp44 would confound the interpretation of the results.

DISCUSSION

In this study, we uncovered a close link between ER function and blood pressure through the ER-resident protein ERp44 and showed that ERp44 is required for suppressing the release of excess ERAP1 into the bloodstream in order to prevent unfavorable hypotension.

Although it was known that ERAP1 digests angiotensin II in vitro (Hatton et al., 2000), localization of endogenous ERAP1 within the ER (Šarić et al., 2002) made its physiological activity ambiguous in vivo. Our data establish an ERp44-mediated, redox-dependent mechanism for controlling intra- and extracellular ERAP1 levels and demonstrate a close link between the ER redox state and blood pressure control. We propose that this mechanism acts in concert with the already-known regulation of angiotensin II in the circulatory system to regulate blood pressure homeostasis. A recent study showed the pH-dependent quality control of secretory proteins by the reciprocal translocation of ERp44 between the ER and Golgi (Vavassori et al., 2013); thus, pH, in addition to redox state, within the ER might also affect blood pressure by ERp44-dependent retrieval of ERAP1 to the ER.

The redox state of the ER is thought to affect protein folding and secretion through the formation of inter- and intramolecular disulfide bonds (Elggaard and Ruddock, 2005; Hatahet and Ruddock, 2009; Margittai and Sitia, 2011; Sevier and Kaiser, 2008), and the effects of deletion of ER-resident oxidoreductases and chaperones on protein secretion and oligomerization have been extensively studied using siRNA-knockdown cells or knockout mice. Zito et al. recently produced Erp1−/− and -β
double-deficient mice and found a selective role for Ero1-β in insulin processing despite the co-expression of Ero1-α in pancreas (Zito et al., 2010a). In addition, although previous work in cultured cells suggested a role for both Ero1 paralogs in immunoglobulin (IgM) assembly in cultured cells (Mezghrani et al., 2001), IgM oxidative folding and secretion were not significantly affected in the double-mutant mice. Thus, although there may be some explanations for the discrepancies (e.g., transient versus stable knockout; redundant mechanism, e.g., Prdx4; Zito et al., 2010b), ER redox states are differently controlled between cultured cells and animals.

Experiments using RNAi knockdown and ERp44-overexpressing cells suggested a role for ERp44 in adiponectin secretion and IgM oligomerization (Anelli et al., 2002, 2003; Long et al., 2010; Phillips et al., 2009; Wang et al., 2007, 2008). Unlike these observations, our preliminary data suggest that adiponectin and IgM secretion and oligomerization are not significantly affected in adult Erp44−/− mouse plasma compared to Erp44+/+ mice (our unpublished data). Thus, the regulation of adiponectin and IgM oligomerization and secretion are likely to differ between cultured cells and mice and might be regulated by other ER-resident molecules in addition to ERp44 in vivo.

Wang et al. recently reported that Erp44−/− mice (129svEv mice backcrossed to C57BL/6 mice for seven generations) exhibited significant embryonic lethality similar to our findings (Wang et al., 2014). However, unlike our study, the few Erp44−/−
mice that survived showed decreased lifespan and died around 8–9 months of age. Additionally, at 6 months of age, the surviving Erp44+/− mice had a slightly abnormal cardiac morphology with enlarged cardiac cells and mild fibrosis and exhibited decreased cardiac function, although the cellular mechanism by which Erp44 deficiency causes these phenotypes in vivo was not explored. We examined the heart histology of 6-month-old FVB × C57BL/6 Erp44−/− mice but could not observe any difference compared with Erp44+/+ and Erp44−/− mice (Figure SSF).

Since regulation of angiotensin concentration is critical for cardiac and kidney structure (Wu et al., 2011) and angiotensin deficiency and hypotension are directly linked to cardiac malfunction and dilated cardiomyopathy (Walther et al., 2004), a prolonged decrease of angiotensin II level in the blood, which we demonstrated in this study, might underlie the cardiac abnormalities of the Erp44−/− mice in their study. Alternatively, differences in the genetic backgrounds of the mice may explain the different phenotypes between our mice and those examined by Wang and colleagues.

The redox state of the ER is influenced by many factors, including normal cellular activities and stress to the organism, such as metabolic diseases. ER stress (Hotamisligil, 2010), which is often accompanied by the accumulation of intracellular oxidants (Santos et al., 2009) and changes to the redox conditions in the ER lumen, may perturb ERAP1 retention in the ER. We demonstrated that upon systemic inflammation, the association of Erp44-ERAP1 increases to prevent undesired hypotension in mice. Given the two distinct physiological roles of ERAP1, within the ER and outside of cells, a better understanding of the regulation of its interaction with ERp44 should be explored as a potential therapeutic target. Since ERAP1 polymorphisms are associated with hypotension (Yamamoto et al., 2002), ankylosing spondylitis (Burton et al., 2007; Hattori et al., 2000; MakSYMowych et al., 2009), and psoriasis (Sun et al., 2010), further studies on the regulation of the ERAP1-ERp44 association and development of specific drugs targeting ERAP1 activity may contribute to treatment of these diseases.

EXPERIMENTAL PROCEDURES

Targeting Vector and Generation of Erp44−/− Mice
Erp44−/− mice were generated by homologous recombination. Figure S1A shows the targeting vector. For full details, see Supplemental Experimental Procedures. For generation of F2 mixed genetic background Erp44 mutant mice, F1 FVB/NJ × C57BL/6 Erp44−/− mice were produced by mating C57BL/6 Erp44−/− with FVB/NJ Erp44+/− mice, and F1 FVB/NJ × C57BL/6 Erp44−/− mice were crossed to generate F2 FVB/NJ × C57BL/6 Erp44+/+, Erp44−/−, and Erp44+/− mice (Figure S1E). F2 Erp44+/− mixed background mice were used in all experiments except for Figures 1A and 1B and Figures S1C–S1F and S1I. Animals were handled according to the guidelines of the RIKEN Brain Science Institute animal experiments committee.

Establishment of MEF Cell Culture
C57BL/6 genetic background MEF cell culture was established by standard methods using embryonic day 12.5 embryos (Lei, 2013). For full details, see Supplemental Experimental Procedures.

DNA Constructs
Erp44 expression vector construction was described previously (Higo et al., 2005). cDNA encoding ERAP1 was amplified by RT-PCR using mouse liver cDNA as a template and cloned into the mammalian expression vector pcDNA3.1 (Invitrogen). For full details of DNA constructs, see Supplemental Experimental Procedures.

Measurement of LAP Activity in Culture Medium
Appropriate volumes of cell culture medium were incubated with L-leucyl-p-nitroanilide-HCl as a substrate (Wako) in 40 mM Tris-HCl buffer (pH 7.5) at 37°C for various durations. Optical density was measured at 405 nm. Endogenous LAP activity in the media of HeLa and MEF cells was measured by treating cells with 300 U/ml of IFN-γ for induction of ERAP1 expression (Serwold et al., 2002).

Depletion of ERAP1 from Mouse Plasma and MEF Cell Culture Medium
The rabbit anti-ERAP1 antibody (ERAP1-c; for full details, see Supplemental Experimental Procedures) and rabbit IgG were covalently linked to affinity beads according to the manufacturer’s instructions (Sumitomo Bakelite). Ten microliters of mouse plasma was diluted 20× with 40 mM Tris-HCl (pH 7.5). 200 μl of culture medium or diluted plasma was applied to the columns (Cosmospin Filter H, Nacalai Tesque) containing ERAP1-c or IgG cross-linked to affinity beads. After rotation overnight at 4°C, the column was centrifuged for 1 min at 10,000 rpm, and the filtrate was used for the aminopeptidase assay using L-leucyl-p-nitroanilide-HCl.

Measurement of Various Enzymatic Activities in Mouse Serum
To measure LAP, GOT, albumin, UA, BUN, and glucose levels in mouse serum, DRI-CHEM slides (Fujifilm) were used with DRI-CHEM 3500 V (Fujifilm) according to the manufacturer’s instructions.

Transfections, Western Blotting, and Immunoprecipitation
We transfected HeLa cells using Fugene 6 (Roche) with vectors encoding ERAP1 (pcDNA3.1-ERAP1) and hemagglutinin (HA)-tagged ERp44 according to the manufacturer’s instructions. For knockdown experiments, we used Lipofectamine 2000 with siRNA directed against ERp44 (Invitrogen; for details, see Supplemental Experimental Procedures). After 48 hr, cells were washed with cold PBS and lysed with TNE buffer with or without 10 mM NEM, which inhibits disulfide bond dissociation (Anelli et al., 2003). To see the effect of DTT, the cells were treated with 10 mM DTT for 10 min at 37°C before lysis. After centrifuging the lysates, the supernatants were incubated with appropriate antibodies and protein G Sepharose or anti-Flag antibody-conjugated Sepharose (M2-beads, Sigma) at 4°C. After 2 hr, the immunocomplexes were washed three times with TNE containing ±10 mM NEM. The samples were separated by SDS-PAGE and immunoblotted as described previously (Hisatsune et al., 2013). For non-reducing SDS-PAGE, samples were combined with a sample buffer without β-mercaptoethanol and loaded onto a SDS-PAGE gel. The following antibodies were used: rabbit anti-ERAP1 (ERAP1-c), rabbit monoclonal anti-ERAP1 (Gene Tex), rabbit anti-ERAP1 (K467, Bio) world), rabbit anti-ERAP4 (described in Supplemental Experimental Procedures), mouse anti-β-actin ( Sigma), rat anti-GRP78 (B6-E6, Santa Cruz), rat anti-HA (3F10, Roche), and rabbit anti-PDI (H-160, Santa Cruz).

For mouse analysis, animals were deeply anesthetized with isoflurane and killed by cervical dislocation. Tissues were removed and homogenized in a 0.32 M sucrose buffer (0.32 M sucrose, 5.0 mM HEPES-NaOH [pH 7.5], 1× proteinase inhibitors; Roche) ±10 mM NEM. Homogenates were centrifuged at 1,000 × g for 15 min. The protein concentration of the supernatant was measured, and the same amount of the proteins was further centrifuged at 100,000 × g for 30 min at 4°C. The pellets were lysed with TNE buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.0% Nonidet P-40, and 1 mM EDTA) ±10 mM NEM for 1 hr at 4°C. The lysates were centrifuged at 15,000 rpm for 10 min at 4°C. Then, we added 5–10 μg of rabbit anti-Erp44 antibody or rabbit IgG and 40 μl of 50% suspension of protein G-Sepharose to the lysates and rotated overnight at 4°C. The protein complexes were washed with TNE buffer five times and were lysed with sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4.0% SDS, 10% β-mercaptoethanol, and 0.1% bromphenol blue). The samples were separated by SDS-PAGE gel, and the gel was used for immunoblotting and silver staining (Silver Stain MS kit, Wako).
To quantify band intensity on western blots, we obtained membrane images with LAS-4000 (Fuji film) and used Multi Gauge software (Fuji film) or ImageJ to quantify band intensities.

For quantification of ERAP1 expression, mouse plasma (0.25 μl) was separated by 7.5% SDS-PAGE, transferred to a membrane, and probed with anti-ERAP1 antibody (Gene Tex). After western blotting, the membrane was stained with amido black to confirm the amount of proteins loaded in each lane.

**In Vitro Angiotensin II Degradation Assay**

Twenty microliters of the MEF cell lysate or 40 μl of water instead of the enzyme mix. The reaction was stopped by adding 50 μl 80% acetonitrile and placed on ice for 15 min. After centrifugation at 12,000 rpm for 5 min at 4 °C, the supernatant was diluted with the same volume of 20 mM phosphate buffer (pH 7.4). The reaction products (40 μl) were separated by HPLC (2690 Separations Module, Waters) as described previously, with slight modification (Pelegmi-da-Silva et al., 2002). Details of HPLC are described in Supplemental Experimental Procedures.

**Measurement of Blood Pressure**

The blood pressure of mice was non-invasively measured at room temperature using an MK-2000 ST monitor (Muromachi Kikai) according to the manufacturer’s instructions.

**Quantitative Analysis of Angiotensin in Mouse Plasma**

Mouse plasma (10 μl) from 2- to 12-month-old FVB/N x C57BL/6 hybrid Erp44+/− and litterate control mice was mixed with stable-isotope-labeled angiotensin II, III, and IV as internal standards (10 fmol/μl) and acetonitrile (ACN) containing 0.5% TFA (44 μl). After vortexing, the sample was sonicated for 5 min and then centrifuged at 14,500 rpm for 10 min at 4 °C. For purification of angiotensins, the supernatant was applied onto a handmade TiO2-C18-Stagetip (GL Science and 3M) (Ishihama et al., 2006) and centrifuged at 6,000 rpm for 3 min. Angiotensins were eluted from the TiO2-C18-Stagetip by centrifugation (6,000 rpm, 3 min two times) using a total of 400 μl 80% ACN/0.1% TFA. The eluent was dried in a speed vacuum and redissolved in 100 μl of 10% ACN containing 0.1% TFA. Desalting was performed on a C18-StageTip and the eluent (50 μl of 70% ACN/0.1% TFA) was dried. The purified peptide fraction containing angiotensins dissolved in 9 μl of 10% ACN/0.1% TFA was used for liquid chromatography MS/MS analysis. The angiotensins were separated on an EASY-nLC 1000 using a NANO-HPLC capillary column C18 (Nikkyo Technos) with a trap column and analyzed online by triple-quadrupole MS (TSQ Vantage EMR, Thermo Fisher Scientific) using a multiple reaction monitoring (MRM) method. An MRM transition list was created for angiotensins (II, III, and IV) and isotope-labeled internal standards by Pinpoint software (Thermo Fisher Scientific). The absolute angiotensin quantitation in plasma was carried out with each stable-isotope-labeled angiotensin by Quan Browser Software (Thermo Fisher Scientific).

**Deglycosidase Treatment**

Nine microliters of the MEF cell lysate or 40×-diluted Erp44−/− mouse plasma was treated with protein deglycosylation mix (NEB) for 4 hr at 37 °C according to the manufacturer’s instructions. For the control, we added the same amount of water instead of the enzyme mix. The reaction was stopped by adding 5 μl of 4× SDS-PAGE sample buffer.

**Induction of Sepsis by IFN-γ and LPS Intraperitoneal Injection in Mice**

Mice (7–8 weeks old, C57BL/6) were intraperitoneally injected with 1.0 μg IFN-γ (Pepro Tech). After 2 hr, LPS (4 mg/kg, Sigma) was further injected as reported previously (Zhang et al., 2003).

**Cell Fractionation**

Cells were homogenized in 0.32 M sucrose buffer. After centrifugation at 1,000 x g for 10 min at 4 °C, the supernatant (S1 fraction) was centrifuged at 100,000 x g for 30 min at 4 °C. The resultant supernatant was the S2 fraction, and the pellet was the P2/P3 membrane fraction.

**Real-Time PCR**

RNA was purified from mouse tissues using RNeasy Mini kit (QIAGEN). cDNA was synthesized using RevertAid Ace qPCR Master Mix (TOYOBO), and real-time PCR was performed using Power SYBER Green PCR master mix (TOYOBO) and Applied Biosystems 7900HT. For quantification, the target mRNA was normalized to the Gapdh mRNA. The primers are described in Supplemental Experimental Procedures.

**Statistical Analysis**

The statistical significance was defined as p < 0.05 using Student’s t test, Mann-Whitney U test, or one-way ANOVA with Bonferroni or Turkey’s multiple-comparison post hoc test, as appropriate.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.04.008.

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