Overload of Neprilysin in Placental Extracellular Vesicles Disrupts CNP-NPRB-Mediated Communication between Vascular Endothelial and Smooth Muscle Cells: A Trigger for Symptoms of Preeclampsia

Running title: NEP in placental EVs triggers hypertension in PE

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ABSTRACT

Background: Preeclampsia (PE) is a placenta-origin pregnancy complication. Although its development has long been divided into two stages: abnormal placentation (stage I) and the release of factors from the hypoperfused placenta into circulation, triggering PE due to endothelial dysfunction (stage II), the placenta-derived substances coupling the two stages remain unclear.

Methods: Extracellular vesicles (EVs) from normal and PE-complicated placentas were intravenously administered to pregnant mice, and blood pressure was recorded throughout pregnancy. The differential cargo, including neprilysin (NEP), of placental EVs in normal and preeclamptic placentas was identified by LC-MS, and the cell types involved in NEP expression in the placenta were determined by single-cell RNA-seq. The effects of placental EVs and recombinant mouse NEP (r-mNEP) on the uterine arteries were assessed by myography. Placenta-specific NEP overexpression mice were established by *in situ* injection of adenovirus. The binding affinity between NEP and the vasodilative peptides was determined using an Octet instrument. NEP-OE HUVECs were established to measure C-type natriuretic peptide (CNP) release and cocultured with natriuretic peptide receptor-B (NPRB)-KD VSMCs to measure cGMP production in VSMCs.

Results: EVs from PE-complicated placentas (PEPL-EVs) impaired vascular endothelium-dependent vasodilation and induced PE in mice. NEP was expressed predominantly by syncytiotrophoblasts and upregulated in PEPL-EVs. r-mNEP administration resulted in outcomes like those of PEPL-EV administration. Placenta-specific NEP overexpression disturbed maternal hemodynamics, resulting in hypertension and proteinuria of the mice. CNP exhibited high binding affinity for NEP, and NEP upregulation in HUVECs inhibited

CNP release, which further influenced the production of cGMP in VSMCs; however, this effect was largely blunted in NPRB-deficient VSMCs

Conclusions: Excessive NEP in PEPL-EVs is transported into the endothelial cells of uterine and placental arteries to cleave and degrade CNP, resulting in compromised CNP paracrine activity and NPR-B-mediated cGMP production in adjacent VSMCs and triggering the hypertensive manifestation of PE.

KEY WORDS: extracellular vesicles, neprilysin, preeclampsia, C-type natriuretic peptide, endothelial dysfunction

Non-standard Abbreviations and Acronyms

ADV-NC	Negative control of adenovirus
ADV-NEP	Adenovirus expressing neprilysin
NEP-OE	Neprilysin overexpression
NPL-EV	Placental extracellular vesicles from normal pregnancies
NPRB-KD	Natriuretic peptide receptor-B knockdown
NPRB-NC	Natriuretic peptide receptor-B negative control
PEPL-EV	Placental extracellular vesicles from preeclamptic pregnancies
r-mNEP	Recombinant mouse NEP

INTRODUCTION

Preeclampsia (PE) is a gestation-specific syndrome characterized by new-onset hypertension, proteinuria, endothelial dysfunction and placental defects and remains the leading cause of neonatal and maternal morbidity and mortality worldwide[1]. Numerous studies have shown that the clinical features of PE are triggered by factors released from the ischemic placenta, which is attributed to impaired spiral arteries remodeling[1,2]. However, current clinical practices for managing PE remain limited to symptomatic therapy (e.g., magnesium sulfate treatment, general antihypertensive treatment) or pregnancy termination, as PE-specific therapies are still lacking[3].

The human placenta produces extracellular vesicles (EVs) throughout gestation, and these EVs can be detected as early as 6 weeks of gestation. Once released, placental EVs (PL-EVs) are deported via the uterine vessel into the maternal circulation, through which they are involved in fetomaternal immune tolerance and are critical for pregnancy[4]. However, emerging evidence suggests a role for PL-EVs in PE pathogenesis[1,4,5].Firstly, an increased number of PL-EVs are found in the peripheral blood of patients with PE compared with those with normal pregnancies[6,7]. Secondly, proteins involved in endothelial injury and the inflammatory response are enriched in PL-EVs from PE-complicated pregnancies[8-10]. However, whether the cargo of PL-EVs directly induces or potentiates the clinical manifestations of PE has yet to be revealed.

Neprilysin (NEP), a neutral endopeptidase, is also known as common acute lymphoblastic leukemia antigen (CALLA), neutrophil antigen cluster differentiation antigen 10 (CD10)

and membrane metalloendopeptidase (MME). This molecule plays an important role in the degradation of a variety of vasoactive peptides, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP)[11]. By promoting vasodilatation and preventing sodium retention and cardiac remodeling, NEP inhibitors combined with angiotensin inhibitors are currently considered novel promising treatments for hypertension and heart failure[12]. Although NEP levels were shown to be greater in the placenta and EVs isolated from the placental perfusate of PE-complicated pregnancies than in those of normal pregnancies[13], the role of NEP in the pathophysiology of PE has yet to be fully elucidated.

The pregnancy-associated reduction in blood pressure has been largely attributed to elevated endothelium-derived vasodilators, including nitric oxide and endothelium-derived hyperpolarizing factor (EDHF), which stimulate relaxation of adjacent vascular smooth muscle cells (VSMCs) via various receptors[14]. Intriguingly, such crosstalk between the endothelium and VSMCs is largely weakened in PE, which may be causative for the impaired vascular adaptation and clinical features of PE[15].

CNP is commonly considered to be released in an autocrine-paracrine fashion from endothelial cells, and it acts as a vasodilator by intracellular cGMP accumulation through the activation of its receptor natriuretic peptide receptor-B (NPR-B)[16]. The expression pattern of CNP suggests a possible effect on controlling vascular tone during pregnancy, as it is highly expressed in human and mouse placentas, with the strongest expression occurring around maternal blood vessels[17,18]. Although maternal CNP plasma

concentrations do not differ between women with normal pregnancies and those with PE[19], given that NPR-B has been identified at high concentrations in adjacent vascular smooth muscle cells[20], the local regulation of CNP within the vascular wall during PE remains unclear.

In this study, we identified NEP as the protein cargo in PL-EVs, which couples the two stages of PE development by disrupting endothelial CNP-dependent VSMC relaxation and thus leads to the onset of clinical manifestations of PE.

Materials and Methods

The data and materials described in the manuscript will be available upon reasonable request made to the corresponding authors, delivery charges and agreement of usage may apply. The detailed materials and methods can be found in the Supplemental Material. Please see the Major Resources Table in the Supplemental Materials. The exact P values are displayed in supplemental materials.

RESULTS

PEPL-EV administration induced PE-like symptoms in pregnant mice

Human term placental EVs were extracted from normal and preeclamptic pregnancies, and the morphology of PL-EVs was characterized by transmission electron microscopy (TEM) and nanoparticle analysis and then verified by the expression of CD9, CD63, ALIX and PLAP (Figure S1A-C). Next, pregnant mice were intravenously administered placental EVs (250 μg total protein) from normal pregnancies (NPL-EVs) or preeclamptic

pregnancies (PEPL-EVs) suspended in 200 μL saline or the supernatant of the final wash (200 μL) once a day from E9.5 to E13.5 (Figure 1A). Compared with those in the NPL-EV or supernatant treatment group, pregnant mice infused with PEPL-EVs exhibited a significant increase in mean systolic blood pressure from E11.5 to E18.5 (Figure 1B). In addition, urinary albumin levels within 24 h were significantly higher in the PEPL-EV treatment group than in the NPL-EV or supernatant treatment group (Figure 1C). Consistently, the glomerular open capillary areas in the kidneys of the PEPL-EV-treated pregnant mice were reduced compared with those in the kidneys of the pregnant controls (Figure 1D-E). In addition, fetal and placental weights, fetal crown-rump length (CRL) and the number of live fetuses were all significantly compromised by treatment with PEPL-EVs (Figure 1F-I). In contrast, the ratio of sFlt-1 to PIGF in the serum and the ratio of the labyrinth zone (LZ) to the junction zone (JZ) of the placenta significantly increased (Figure 1J-N). Nevertheless, the administration of PEPL-EVs to nonpregnant female mice resulted in neither hypertension nor proteinuria (Figure S2A-C). Accordingly, glomerular enlargement was also absent in the nonpregnant mice treated with PEPL-EVs (Figure S2D-E).

Inhibition of EV uptake abolished the PEPL-EV-induced PE-like symptoms in pregnant mice

To identify the recipient cell types of PL-EVs *in vivo*, PKH67-labeled PL-EVs were intravenously infused into pregnant mice on E12.5, along with 15 mg/kg heparin, a blocker of EV internalization[21,22], or an equal volume of PBS. PKH67-stained PL-EVs were deposited mainly in the placenta rather than in the liver or kidneys (Figure S3A-D).

Specifically, PL-EVs colocalized with CD31-positive endothelial cells in the labyrinth and spiral arteries (Figure 2A). However, such colocalization in the placenta was largely inhibited in the presence of heparin, strongly suggesting the uptake of PL-EVs by endothelial cells (Figure S3C). Our *in vitro* data further confirmed the uptake of PL-EVs by HUVECs in a time-dependent manner. Similarly, nearly 60% of the internalization of PL-EVs by HUVECs was blocked in the presence of heparin (Figure 2B and Figure S4). Most importantly, engulfment of PKH67-stained PL-EVs by the endothelial cells of maternal uterine arteries in living mice was visualized by two-photon microscopy (Figure 2C), whereas injection of the final wash of PKH67-stained EVs did not result in detectable fluorescence in the placenta, kidneys, liver or uterine arteries (Figure S5A-C). These findings strongly suggest that PL-EVs are taken up mainly by endothelial cells in the placenta and maternal uterine arteries.

Furthermore, our results demonstrated that the significant increase in systolic blood pressure induced by PEPL-EVs was abolished by the coadministration of heparin. Nevertheless, heparin treatment alone did not cause hypotension (Figure 2D), implying that inhibition of EV internalization *in vivo* rescues PEPL-EV-induced PE-like symptoms in mice. Consistent with this finding, administration of heparin resulted in lower levels of urinary albumin (Figure 2E). Moreover, heparin prevented glomerular enlargement and glycogen deposition in the kidneys induced by PEPL-EVs (Figure S6A). Additionally, heparin partially restored fetal BW, fetal CRL and the number of live fetuses, all of which were compromised by PEPL-EV treatment alone (Figure S6B-E). Notably, in addition to endocytosis, EVs may enter cells via lipid membrane fusion, which was assessed by fusion

assays based on R18 lipid probe dequenching. Our data revealed that membrane fusion continuously occurred even in the presence of heparin, explaining the partial blockade of EV-mediated intercellular cargo transport by heparin (Figure 2F). Thus, PEPL-EV-induced PE manifestations are dependent on internalization by endothelial cells.

PEPL-EVs impaired endothelium-dependent relaxation of uterine arteries in pregnant mice

PE has long been associated with vascular dysfunction, which is characterized by impaired endothelium-dependent relaxation. To determine whether PL-EVs are involved in the regulation of vascular function *in vivo*, PL-EVs (250 μg total protein) suspended in 200 μL saline were intravenously infused into pregnant mice daily from E9.5 to E13.5, and the uterine arteries were then dissected on E18.5 and subjected to e*x vivo* assessment of vascular function by wire myography. Compared with exposure to NPL-EVs or supernatant, exposure to PEPL-EVs significantly impaired the relaxation of uterine arteries in response to acetylcholine (Figure 3A and Figure S7A). Intriguingly, pre-administration of heparin partially but significantly prevented the impairment of vasorelaxation previously observed in the arteries exposed to PEPL-EVs alone, whereas saline or heparin alone did not interfere with vascular dilation in response to acetylcholine (Figure 3B).

Nitric oxide (NO) has previously been reported to have major effects on the vasodilatory response in arteries from pregnant subjects[23,24]. To determine whether PEPL-EVs compromise vasorelaxation by impeding endothelial NO production, first, NO production in HUVECs in response to PL-EVs was evaluated by probing with DAF-FM DA. The data

showed that PEPL-EV treatment did not disturb intracellular NO levels in HUVECs (Figure 3C). Then, Griess reagent was used to detect nitrite, which can form by the spontaneous oxidation of NO under physiological conditions. Neither the coincubation of PEPL-EV nor that of PL-EV obviously disturbed nitrite levels in the culture medium of HUVECs in the presence or absence of heparin (Figure 3D-E). Although impaired endothelial cell proliferation impairs endothelium-dependent vasodilation, neither tube formation nor the survival of endothelial cells was affected by PL-EVs (Figure S8A-B). These results strongly indicate that PEPL-EV-induced dysfunction of endothelium-dependent relaxation in uterine arteries is independent of NO.

PEPL-EVs are overloaded with NEP

EVs mediate intercellular communication by carrying cargos, including messenger RNAs, microRNAs, lipids and proteins. Although the role of PL-EV-loaded small RNAs in endothelial dysfunction in PE has been elucidated, the involvement of the protein cargo of PL-EVs in PE has yet to be identified. Therefore, this study profiled the proteomics of NPL-EVs and PEPL-EVs for the first time. A total of 3641 proteins were identified by LC-MS (Figure 4A). Among them, 27 proteins were found to be significantly different between the two groups (fold change > 2, $P < 0.05$), 13 proteins were enriched in NPL-EVs, and 14 proteins, including NEP, were enriched in PEPL-EVs (Figure 4B). Further validation of NEP upregulation in PEPL-EVs revealed a 3-fold increase in the NEP-to-PLAP ratio in PEPL-EVs compared with that in NPL-EVs ($P = 4.87 \times 10\text{-}5$), whereas the level of placental alkaline phosphatase (PLAP), a biomarker of syncytiotrophoblasts, did not differ (Figure 4C). The upregulation of NEP in PEPL-EVs was further confirmed by NEP-labeled

immunogold transmission electron microscopy (Figure 4D). Similar increases in NEP were observed in the whole lysate of preeclamptic placental tissue (Figure 4E) and further validated by IF staining (Figure 4F). Moreover, NEP was localized predominantly in the CK7-positive trophoblasts of the syncytium rather than in the CD31-positive endothelium. For further identification of the cell types expressing NEP in the placenta, term placentas were collected from three women with normal pregnancies (Table S2) and subjected to 10× single-cell RNA sequencing (scRNA-seq) analysis. Twelve cell clusters were identified via UMAP analyses (Figure S9A-D). As expected, NEP was expressed predominantly by syncytiotrophoblasts but was barely detected in endothelial cells (Figure 4G).

Next, we determined whether syncytiotrophoblastic NEP is transported into endothelial cells via EVs *in vivo*. Pregnant mice were administered PEPL-EVs with or without heparin. IF staining of CD31 and NEP in uterine arteries confirmed that infusion of PEPL-EVs markedly elevated NEP enrichment in the endothelium of uterine arteries, but this change was largely diminished in the presence of heparin (Figure S10A). Accordingly, *in vitro* evidence revealed that incubation with PEPL-EVs increased NEP protein levels in HUVECs in a dose-dependent manner, which was blocked by heparin (Figure S10B). Importantly, the enzymatic activity of NEP encapsulated in PEPL-EVs was comparable to that of NEP encapsulated in NPL-EVs (Figure S11), indicating that PEPL-EV-induced vascular dysfunction and consequent PE symptoms might be attributed to excessive NEP transport via EVs from syncytiotrophoblasts to endothelial cells, which lack endogenous NEP.

Placenta-specific overexpression of NEP induced PE-like symptoms in mice

To mimic the elevation of NEP in placental tissue and PL-EVs in PE, placenta-specific NEP-overexpressing mice were generated by *in situ* NEP overexpression (Figure 5A-B). As shown in the results, NEP protein levels in placental tissue were increased by 50% compared with those in controls (Figure 5C). Importantly, the EVs isolated from both the placental tissue and peripheral blood of Adv-NEP mice contained significantly higher levels of NEP protein than the control groups did (Online Figures S12 and S13). Similar to women with PE-complicated pregnancies, Adv-NEP mice presented a pronounced increase in systolic blood pressure from E15.5 to E17.5 compared with control mice (Figure 5D). The ultrasonic results revealed that the resistive index (RI) and pulsatile index (PI) of the uterine arteries were notably higher in the Adv-NEP mice than in the other mice, indicating increased blood flow resistance. Moreover, the systolic/diastolic (S/D) ratio in the Adv-NEP mice was approximately 1.5 times greater than that in the Adv-NC and control mice, indicating substantial alterations in arterial wave dynamics and blood flow characteristics (Figure 5E). Accordingly, Adv-NEP mice presented a 2-fold increase in urinary albumin excretion and a 50% reduction in the glomerular vascular open area compared with the Adv-NC and control mice, implying compromised renal microvasculature and function (Figure 5F-H). Consequently, Adv-NEP mice presented significantly reduced fetal BW, CRL, and number of life fetuses and increased ratios of sFlt-1 to PIGF and LZ to JZ ratio, while the difference in placental weight across mice in treatment groups was subtle (Figure 5I-Q).

Excessive NEP impaired vasorelaxation and caused hypertension in pregnant mice

To further validate the detrimental role of NEP in vasorelaxation, pregnant mice were intravenously administered r-mNEP (400 ng total protein in 200 μl) daily from E9.5 to E13.5. The administration of r-mNEP resulted in a significant increase in the mean systolic blood pressure from E11.5 to E18.5, whereas treatment with denatured NEP (95 °C for 10 min; designated r-mNEP 95 °C) did not disturb blood pressure (Figure 6A). In addition, treatment with r-mNEP rather than r-mNEP 95 °C led to a significant increase in the resistance index (RI), pulsatility index (PI) and systolic/diastolic (S/D) ratio of the uterine arteries, as measured on E18.5 by Doppler ultrasonography (Figure 6B-C and Figure S14A-B). Furthermore, the wire myography results revealed that, compared with those from the r-mNEP 95 °C-treated and control mice, the uterine arteries from r-mNEP-treated mice were associated with blunted endothelium-dependent vasodilation in response to acetylcholine (Figure 6D). Interestingly, r-mNEP had no effect on perinatal outcomes, including fetal BW, placental weight, CRL or number of life fetuses (Figure 6E-H). Taken together, these results strongly suggest that abnormally elevated NEP in syncytiotrophoblasts (STBs) can be transported into the endothelium, which is responsible for impaired vasorelaxation and consequent hypertension.

NEP primarily bound with and degraded CNP in endothelial cells

Since NEP cleaves a variety of vasodilative peptides, including bradykinin, ANP and C-type natriuretic peptide (CNP), as well as the vasoconstrictive peptides ET-1 and Ang II, its overall physiological role in the regulation of vascular function remains unclear. Here, the binding affinities between the vasoconstrictive peptides and NEP were assessed via

gradient experiments. Serial dilutions (3.125–100 μM) of ANP, ET-1, CNP, Ang II and bradykinin peptides were incubated with NEP. ANP, ET-1 and CNP all bound to the immobilized NEP protein in a dose-dependent manner. For Ang II and bradykinin, incubation with only the two highest concentrations resulted in binding to NEP (Figure 7A-E). The equilibrium dissociation constant (K_D), which represents the strength of the binding affinity of the vasoconstrictive peptide-NEP interaction was then calculated as off-rate kinetic values/on-rate kinetic values (K_{off}/K_{on}). As shown in Table 1, the binding affinity to NEP was ranked CNP > ANP > ET-1 > bradykinin > Ang II. To verify whether increased intracellular NEP reduces CNP release by endothelial cells, we next established NEP-overexpressing (NEP-OE) HUVECs (Figure 7F and Figure S15A), in which the extracellular CNP level was significantly reduced (Figure 7G). Thus, the NEP encapsulated in PEPL-EVs can degrade CNP in endothelial cells.

Table 1: Binding kinetics of NEP to ANP, ET-1, CNP, Ang II and bradykinin.

Peptides	**K_D (M)**	**K_{on} (1/Ms)**	**K_{off} (1/s)**
ANP	1.60E-05	5.98E+04	9.59E-01
ET-1	9.84E-06	5.56E+04	5.47E-01
CNP	2.55E-06	1.94E+05	4.95E-01
Ang II	K_D > 1 mM	/	/
Bradykinin	K_D > 1 mM	/	/

The off-rate kinetics (K_{off}) were measured by saturating the SSA with NEP and monitoring the dissociation after the SSA was switched to buffer. The on-rate kinetics (K_{on}) were measured using different concentrations of ANP, ET-1, CNP, Ang II and bradykinin. K_D,

the equilibrium dissociation constant, was calculated as K_{off}/K_{on} for ANP, ET-1, CNP, Ang II and bradykinin.

Endothelial CNP stimulated cGMP production in VSMCs through natriuretic peptide receptor-B

CNP is an important endothelium-derived vasorelaxant. This molecule modulates vascular tone by specifically binding to NPR-B rather than to natriuretic peptide receptor-A (NPR-A) on vascular smooth muscle cells (VSMCs), which catalyzes the conversion of GTP to the second messenger cGMP and results in vasodilation. We further investigated whether NEP from PL-EVs interrupts CNP-natriuretic peptide receptor (NPR)-mediated vasodilation signaling in VSMCs.

Next, a coculture of HUVECs and VSMCs was established (Figure S14B). NEP-OE HUVECs were cocultured with natriuretic peptide receptor-B knockdown (NPRB-KD) and NPRB-negative control (NPRB-NC) A10 cells (Figure S15C). We found that cGMP was significantly increased in the NPRB-NC A10 cells cocultured with NEP-NC HUVECs compared with NEP-OE HUVECs, but this HUVEC-stimulated increase in cGMP was abrogated by NPRB depletion (NPRB-KD) in A10 cells. Furthermore, the overexpression of NEP in HUVECs did not affect cGMP in NPRB-KD A10 cells (Figure 7I). Given that upregulation of NEP in HUVECs degrades CNP, these findings indicate that endothelial CNP stimulates cGMP production in adjacent VSMCs via NPR-B.

Discussion

The pathogenesis of PE classically involves two stages. Specifically, multiple internal or external factors cause abnormal placentation in stage I, and then the hypoperfused placenta releases factors into circulation that trigger the onset of PE, which is attributed mainly to endothelial dysfunction in stage II. However, the placenta-derived substances that couple the two stages of PE development remain poorly understood. Although soluble fms-like tyrosine kinase 1 (sFlt-1) may be involved in this process[25], emerging evidence also suggests that PE development may be associated with the release of EVs from the placenta into the maternal circulation[4,6,9,26]. Data from cellular experiments have demonstrated that EVs isolated from preeclamptic plasma impair the function of endothelial cells, which is attributed to their cargo[10,27]. In this study, we are the first to provide *in vivo* evidence that PEPL-EVs induce hypertension, proteinuria and fetal growth restriction, all of which are hallmark events of PE, in pregnant mice.

EVs derived from different sources have been reported to interact preferentially with specific cell types, especially source cells and/or adjacent cells within the secreting organ[28,29], which may be related to the specific engagement of recipient cell receptors with the EV composition. The present study revealed that PL-EVs were preferentially internalized by endothelial cells in the placenta and maternal uterine arteries of pregnant mice. Moreover, the administration of PEPL-EVs did not induce PE-like symptoms in nonpregnant mice, confirming that the placenta is the target organ of PL-EVs. In contrast to previous work by Chang et al., which focused on plasma exosomes, our study revealed that PL-EVs are internalized at lower levels by the liver and kidneys of pregnant mice.

Given that EV-mediated cell-to-cell communication depends on its cargo transfer to target cells, which is mediated by (i) fusing with the plasma membrane of recipient cells, (ii) various forms of endocytosis, or (iii) breaking down and releasing their cargo, heparin may not be able to fully inhibit EV uptake and membrane fusion. Indeed, although heparin effectively inhibited the uptake of PL-EVs by recipient cells both *in vitro* and *in vivo*, it partially rather than completely reversed PEPL-EV-induced PE-like symptoms in pregnant mice. This finding is likely because PL-EVs can be incorporated into vascular endothelial cells through membrane fusion.

EVs isolated from preeclamptic plasma were shown to inhibit the tube formation of endothelial cells and vasodilatation of isolated vessels[10,30]. This process is related to NO, whose effects on vasodilation have been well characterized, and decreased NO levels in the serum have been identified in preeclamptic endothelia. Unexpectedly, our results demonstrated that PL-EVs have no effect on NO production in cultured endothelial cells or on angiogenesis and viability, suggesting that the impairment of vasodilation by PE-EVs may be independent of NO synthesis. Notably, in addition to NO, endothelium-derived hyperpolarizing factors (EDHFs), including ANP, BNP, and CNP, elicit smooth muscle hyperpolarization and relaxation and are thus considered to trigger endothelium-dependent vasodilation in the resistant vasculature of mammals[31]. However, the role and regulation of EDHFs in PE development remain largely unknown.

By performing unbiased high-throughput proteomics, we identified NEP as one of the cargos discriminating between PEPL-EVs and NPL-EVs, which is consistent with a

previous report by Gill et al.[13] This upregulation of NEP in PE could be attributed to the increase in hypoxia-inducible factor 1α, which has long been associated with preeclamptic placentas. Moreover, we determined that NEP is exclusively expressed by STBs, which directly contact maternal blood and produce and release many EVs during pregnancy[32]. These findings strongly imply that STBs may play a critical role in fetomaternal communication by delivering substances such as NEP to maternal cells through EVs. Since NEP cleaves and inactivates a variety of vasoactive peptides, including EDHFs[11], excessive NEP encapsulated in PEPL-EVs may suppress the bioavailability of EDHFs in the vascular bed of pregnant women, triggering endothelial dysfunction and consequent hypertension of patients with PE. However, to the best of our knowledge, the causative role and underlying mechanisms of NEP in PE development have yet to be elucidated.

In the present study, we show that either specific upregulation of placental NEP or administration of r-mNEP could induce PE-like symptoms in mice. In addition, the results of myography and Doppler ultrasonography demonstrate that the elevation of NEP in circulation impaired endothelial function and increased vascular resistance in the uterine arteries of pregnant mice. Moreover, we provide both *in vitro* and *in vivo* evidence that PL-EVs enhance the enrichment of NEP in the maternal endothelium. Interestingly, in contrast to PEPL-EV-treated pregnant mice, which exhibit fetal growth restriction and placental maldevelopment, r-mNEP-treated mice did not experience effects on fetal or placental weight. This is likely because NEP specifically triggers endothelial dysfunction in stage II of PE development, whereas other cargos of PEPL-EVs, such as PEG10 (paternally expressed gene 10) and PAPPA2 (pregnancy-associated plasma protein A2), play more

profound roles in the regulation of intrauterine fetal growth and placental development[33]. Moreover, although placenta-specific upregulation of NEP resulted in a reduction in fetal BW, placental weight was maintained. This may be attributed to the relatively late time point of Adv-NEP administration (on E11.5), after the mouse placenta was fully formed.

Although NEP cleaves both vasoconstrictive and vasodilative peptides, our data showed that excessive NEP results in overall vasoconstriction rather than vasodilatation in the uterine arteries of pregnant mice, implying that NEP degrades primarily vasodilative peptides, as plasma CNP levels increase during pregnancy. We therefore generated NEP-OE HUVECs to investigate the role of NEP in the stability of target vasodilative peptides. The data revealed that the upregulation of NEP did not affect the levels of ANP, Ang II, bradykinin or ET-1 in endothelial cells but did affect the level of CNP (Figure S13A-D), as CNP has the highest binding affinity with NEP among these peptides. This finding is in accordance with a previous report that CNP is more rapidly hydrolyzed by NEP than by other natriuretic peptides. Therefore, the upregulation of NEP-induced endothelial dysfunction in PE is possibly dependent on the clearance of CNP and the consequent loss of vasodilation. However, CNP levels in plasma are equivalent between normal and preeclamptic pregnancies, which has been verified by previous reports[34,35]. This is likely because extracellular NEP may interfere with the paracrine activity of CNP in endothelial cells[36,37]. Moreover, the half-life of CNP in blood is very short due to presence of multiple degradative enzymes; therefore, it is difficult to capture fluctuations in circulatory CNP levels via regular detection methods. Indeed, using MALDI-TOF MS, Thonsgaard et al. clearly demonstrated the degradation of cleaved mature CNP rather than pro-CNP by NEP

in vitro[38].

Once secreted, CNP can regulate vascular tone by (i) binding specifically to NPR-B on VSMCs[39], which catalyzes the conversion of GTP to the second messenger cGMP and results in cGMP-mediated vasodilation[34,40], and (ii) binding to natriuretic peptide receptor-C (NPR-C), which opens a G-protein-gated inwardly rectifying K^+ (GIRK) channel, resulting in hyperpolarization. As a locally acting hormone, CNP is released in an autocrine-paracrine fashion. Due to rapid metabolism by clearance receptors and NEP, circulating CNP levels are often at or below the limits of detection. Hence, *in vivo* CNP levels remain to be explored.

Accumulating evidence suggests that vasoconstriction of the uterine arteries can lead to systemic blood pressure elevation via multiple mechanisms: (i) When the uterine arteries experience diastolic dysfunction, blood perfusion fails. The body may thus increase blood pressure to ensure a sufficient uterine blood supply.[41] (ii) Diastolic dysfunction stimulates local receptors. Signals travel to the central nervous system, constrict arterioles, and increase blood pressure.[42] (iii) Endothelial dysfunction manifests as the release of fewer vasodilators or the release of more contracting factors, such as endothelin-1 (ET-1), in PE.[43]Nevertheless, in addition to these direct effects, compromised hemodynamics in the fetomaternal vasculature may also contribute to the occurrence of hypertensive symptoms in preeclampsia through multiple indirect mechanisms. Ischemic hypoxic placenta due to hypoperfusion induces the production of many circulating inflammatory factors and reactive oxygen species, which are known contributors to hypertension

development.[43] Studies on a mouse model of reduced uterine perfusion pressure (RUPP) suggested that elevated RI, PI, and S/D of the uterine arteries due to partial restriction of the lumen can induce systemic hypertension via reduced venous capacitance and compliance, in addition to elevated sFlt-1.[44]

In conclusion, excessive NEP expressed in the STBs of preeclamptic placenta was encapsulated in EVs and transported into the endothelial cells of the uterine arteries and placenta to cleave and degrade CNP, resulting in compromised CNP paracrine activity and NPR-B-mediated cGMP production in adjacent VSMCs, which in turn resulted in vasospasm and an increase in the RI, PI and S/D, ultimately leading to a systemic increase in peripheral blood pressure, the hallmark of PE. Nonetheless, the involvement of NEP from non-PL-EV sources cannot be absolutely ruled out. Other differentially abundant cargos of PEPL-EVs might also couple the two stages of PE development, albeit to a lesser degree.

This study provides a new paradigm for trophoblast-endothelium crosstalk in PE pathogenesis and the ability to predict PE by measuring the NEP level in PL-EVs. There are currently several trials using LCZ696, a novel dual angiotensin-NEP inhibitor, to treat hypertension[12]. Our findings reveal that PE may be a new indication for this drug as well as other NEP inhibitors, although the efficacy and safety for use during pregnancy require further validation.

Acknowledgments

This study was funded by the National Key R&D Program of China (2022YFC2702400), the National Natural Science Foundation of China (U21A20346, 82071675, 81871189, 82101776, 82101711), and the Natural Science Foundation of Chongqing, China (CSTB2022NSCQ-MSX0114).

Disclosures

None.

Supplemental material

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Figures & legends

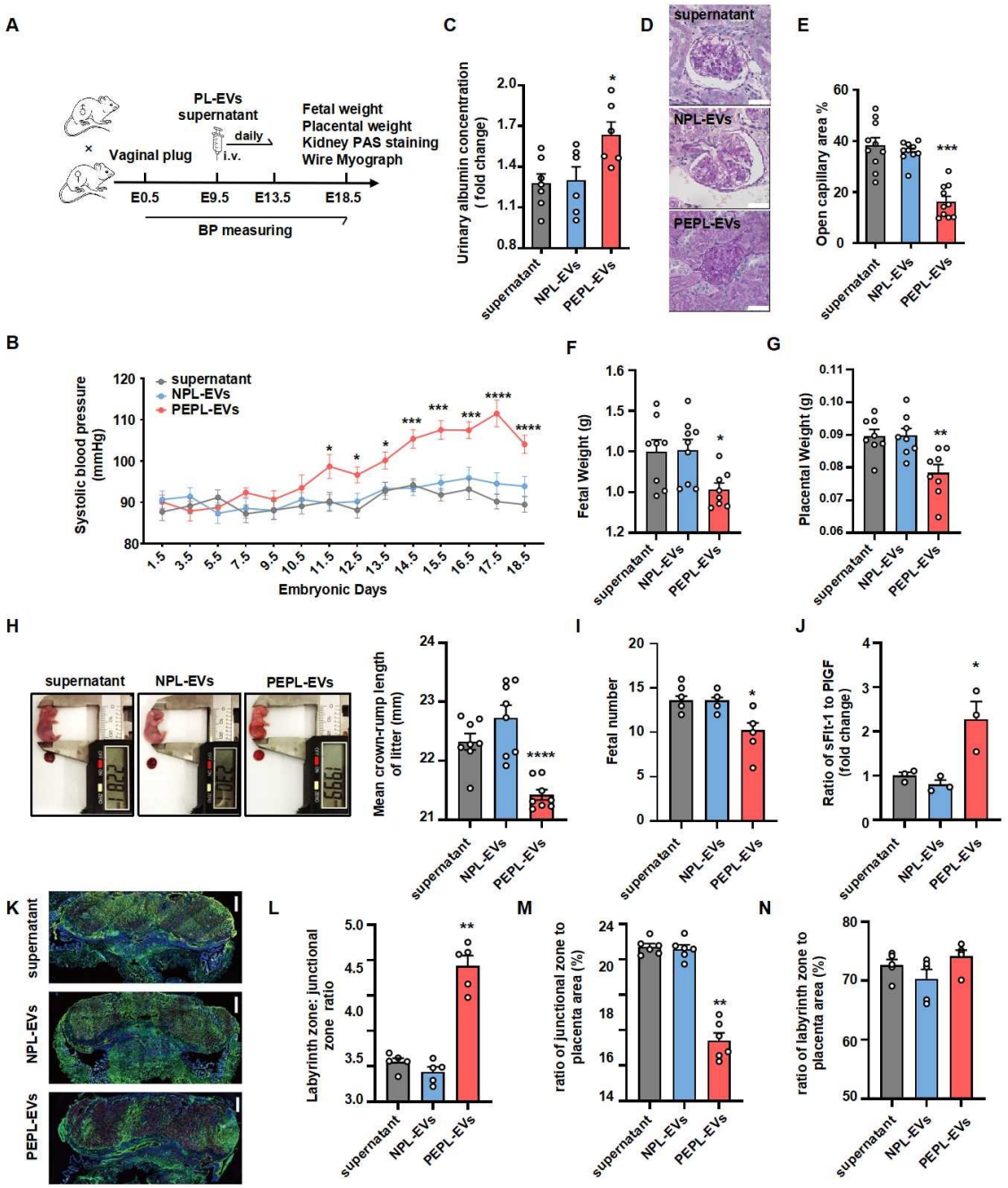

Figure 1. PEPL-EVs induced PE-like symptoms in pregnant mice.

(A) Scheme of the experimental design. (B) Mean systolic blood pressure of the mice throughout pregnancy (n=13 dams in group supernatant and PEPL-EV, n=12 dams in group NPL-EVs). Two-way ANOVA and Tukey's multiple comparison test were used. (C) Fold

change in albumin levels in urine collected from E16.5 to E17.5 (n=7 dams in group supernatant, n=6 dams in group PEPL-EVs and NPL-EVs). (D) Representative PAS staining of kidney sections from dams collected on E18.5 (scale bar: 100 μm). (E) Quantification of the glomerular open capillary area normalized to the glomerular tuft area (n=10 dam). (F) Fetal BW (n=8 dams). (G) placental weight (n=8 dams). (H) CRL (n=8 dams;) and (I) number of live fetuses on E18.5 (n=8 dams). (J) Ratio of sFlt-1 to PIGF in dams on E18.5 (n=3 dams). (K) Immunofluorescence images of the placenta, (L) ratio of the labyrinth zone to the junction zone (n=5 dams). (M) ratio of the junction zone to the placenta area (n=5 dams), and (N) ratio of the labyrinth zone to the placenta area on E18.5 (n=5 dams). One-way ANOVA and Tukey's multiple comparison test were used in C, E-I. The Kruskal-Wallis test and Dunn's multiple comparison were used in J, L-N. The results are presented as the means ± SEMs.

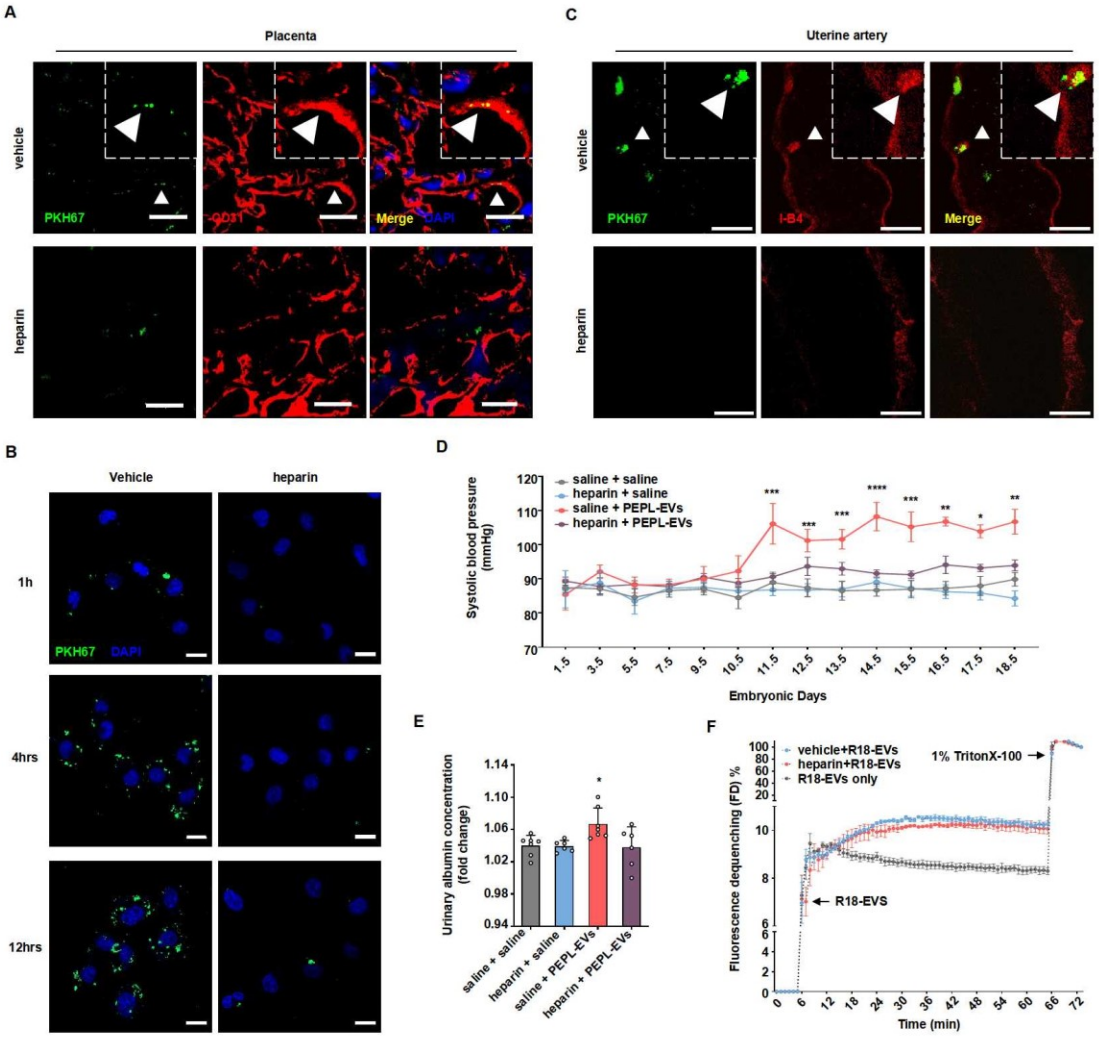

Figure 2. Inhibition of PL-EV uptake by endothelial cells blunted the PEPL-EV-induced PE-like symptoms in pregnant mice.

PL-EVs stained with PKH67 (green) were injected into pregnant mice on E12.5 with or without heparin. (A) Laser scanning confocal fluorescence microscopy images of vascular endothelial cells (CD31: red) in mouse placenta (scale bar: 20 μm) at 12 h post-injection. (B) HUVECs were incubated with PKH67-labeled PL-EVs (green) in the absence or presence of 100 μg/mL heparin for 1, 4 or 12 h (scale bar: 20 μm). (C) Two-photon microscopy images of endothelial cells (IB4: red) in maternal uterine arteries (scale bar: 20 μm) at 12 h post-injection. (D) Mean systolic blood pressure (n=7 dams). (E) Fold

change in urinary albumin levels from E16.5 to E17.5 (n=6 dams in heparin + saline and heparin + PEPL-EVs and n=7 in other groups). (F) PL-EVs prestained with octadecyl rhodamine B (R18) were incubated with HUVECs in the absence or presence of 100 μg/mL heparin, and fusion was monitored for 80 min at 560-nm excitation and 585-nm emission wavelengths using a spectrofluorometer (n=3 samples). One-way ANOVA and Tukey's multiple comparison test were used in E. Two-way ANOVA and Tukey's multiple comparison test were used in D. The results are presented as the means ± SEMs.

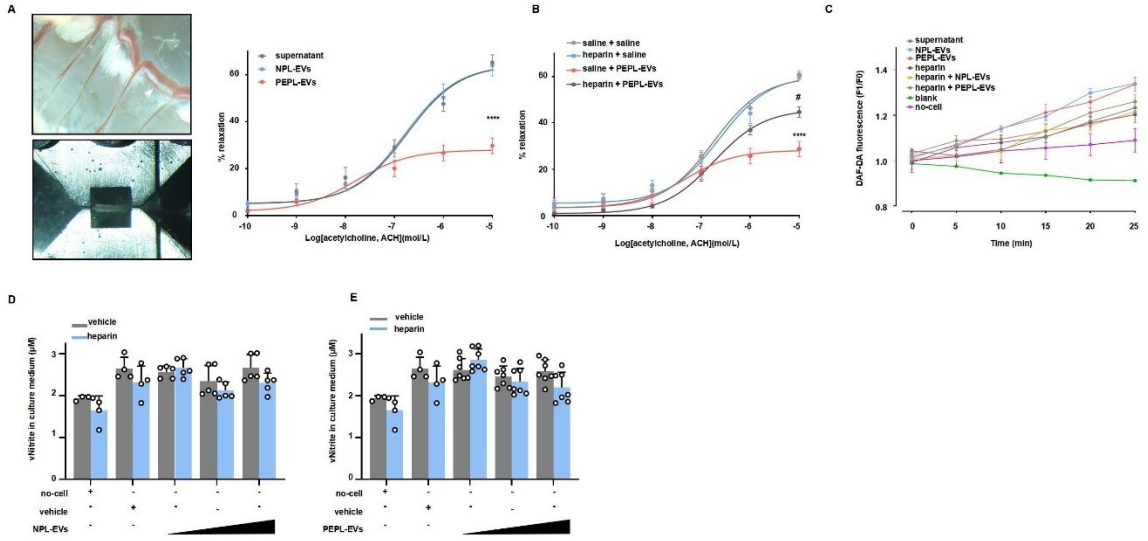

Figure 3. PEPL-EVs impair endothelium-dependent vasodilation of uterine arteries in a non-NO-dependent manner.

(A) Isolated uterine arteries (upper) and arterial rings mounted in the chamber of a myograph (lower). ACh-induced relaxation of uterine arteries from pregnant mice on E18.5 injected with PL-EVs (n=12 dams; Log(Ach)=-5 mol/L) or (B) PEPL-EVs in the presence or absence of heparin from E9.5 to E13.5 (n=6 dams except for group saline + PEPL-EVs where n=7 dams). (C) NO production (n = 3 samples) and (D-E) nitrite levels (n=3 and 4 samples in no-cell + vehicle and no-cell + heparin groups, n=4 vehicle + vehicle and vehicle + heparin groups, n=5 and 7 samples in NPL-EVs related and PE-PL-EVs related groups) in HUVEC culture medium (cell-free, no cells in the well; blank, no treatment). Two-way ANOVA and Tukey's multiple comparison test were used in A-B; Two-way ANOVA and Tukey's multiple comparison tests were used in C-E. The results are presented as the means ± SEMs.

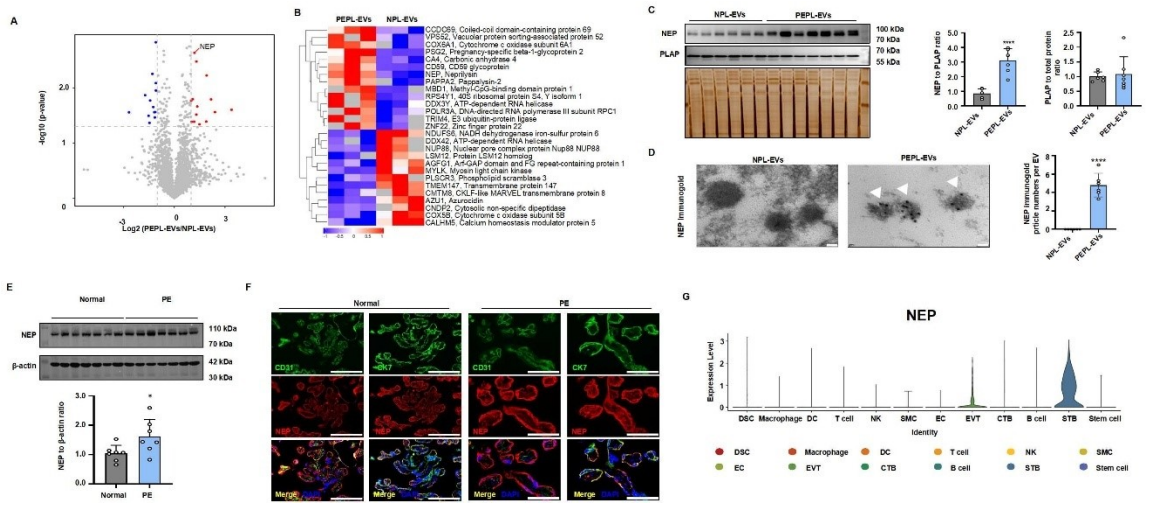

Figure 4. Increased NEP expression in preeclamptic placentas and PEPL-EVs.

(A)Volcano plot and threshold of fold change >2 ($P < 0.05$) and (B) heatmap showing differentially expressed proteins in PL-EVs from normal and preeclamptic placentas. (C) Western blot analysis of NEP and PLAP in PL-EVs from preeclamptic and healthy subjects. Silver staining was used to determine the protein loading amount (n=6 dams in NPL-EVs and 7 dams in PEPL-EVs). Independent t-test was used. (D) NEP-labeled immunogold transmission electron microscopy of PL-EVs (scale bar: 50 nm) (n=3 samples). (E) Placental lysates from preeclamptic and normal placentas were analyzed for NEP expression (n=7 dams;). (F) IF staining of CD31 (green, left panel), CK7 (green, right panel) and NEP (red) in frozen sections of human term placentas; nuclei were stained with DAPI (blue) (scale bar: 100 μm). (G) Violin plots for the expression level of NEP across 12 clusters in normal placentas (DSC, decidual stromal cell; DC, dendritic cell; NK cell, natural killer cell; SMC, smooth muscle cell; EC, endothelial cell; EVT, extravillous trophoblast; CTB, cytotrophoblast; STB, syncytiotrophoblast. Independent t-test was used in C-E. The results are presented as the means ± SEMs.

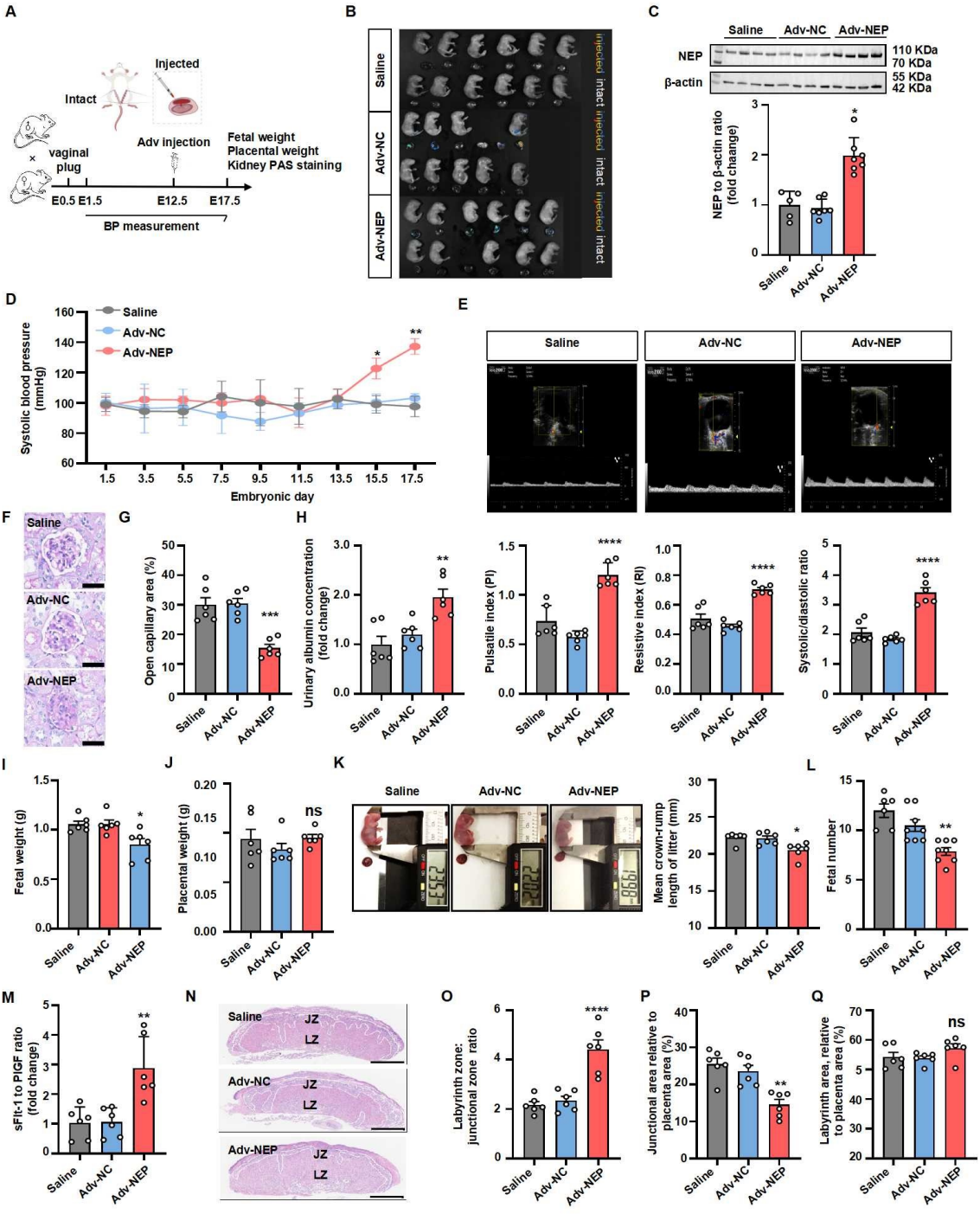

Figure 5. Placenta-specific overexpression of NEP induced PE-like symptoms in mice

(A) Scheme of the experimental design. (B) *Ex vivo* tissue imaging revealed placenta-specific expression of NEP. (C) Western blotting of NEP in mouse placentas (n= 7 dams in Adv-NC and Adv-NEP, N= 5 dams in saline). (D) Mean systolic blood pressure of the

mice throughout pregnancy (n=3 dams). (E) Doppler ultrasound images of the uterine arteries (n=6 dams). (F) Representative PAS staining of kidney sections (scale bar: 100 μm) and (G) quantification of the glomerular open capillary area normalized to the glomerular tuft area (n=6 dams). (H) Albumin levels in pooled urine (n=6 dams). (I) Fetal BW (n=6 dams), (J) placental weight (n=6 dams; ns, nonsignificant vs. saline and Adv-NC), (K) CRL (n=6 dams) and (L) number of life fetuses (n=6 in saline group and n=8 in both Adv-NC and Adv-NEP groups) on E17.5. (M) Ratio of sFlt-1 to PIGF in dams on E17.5 (n=6 dams). (N) HE staining of the placenta (JZ=junction zone, LZ=labyrinth zone), (O) ratio of the labyrinth zone to the junction zone (n=6 dams) , (P) ratio of the junction zone to the placenta area (n=6 dams), and (Q) ratio of the labyrinth zone to the placenta area (n=6 dams; ns, nonsignificant vs. saline and Adv-NC) on E17.5. One-way ANOVA and Tukey's multiple comparison test were used in E, G-M, O-Q; Two-way ANOVA and Tukey's multiple comparison test were used in D; The Kruskal-Wallis test and Dunn's multiple comparison were used in C. The results are presented as the means ± SEMs.

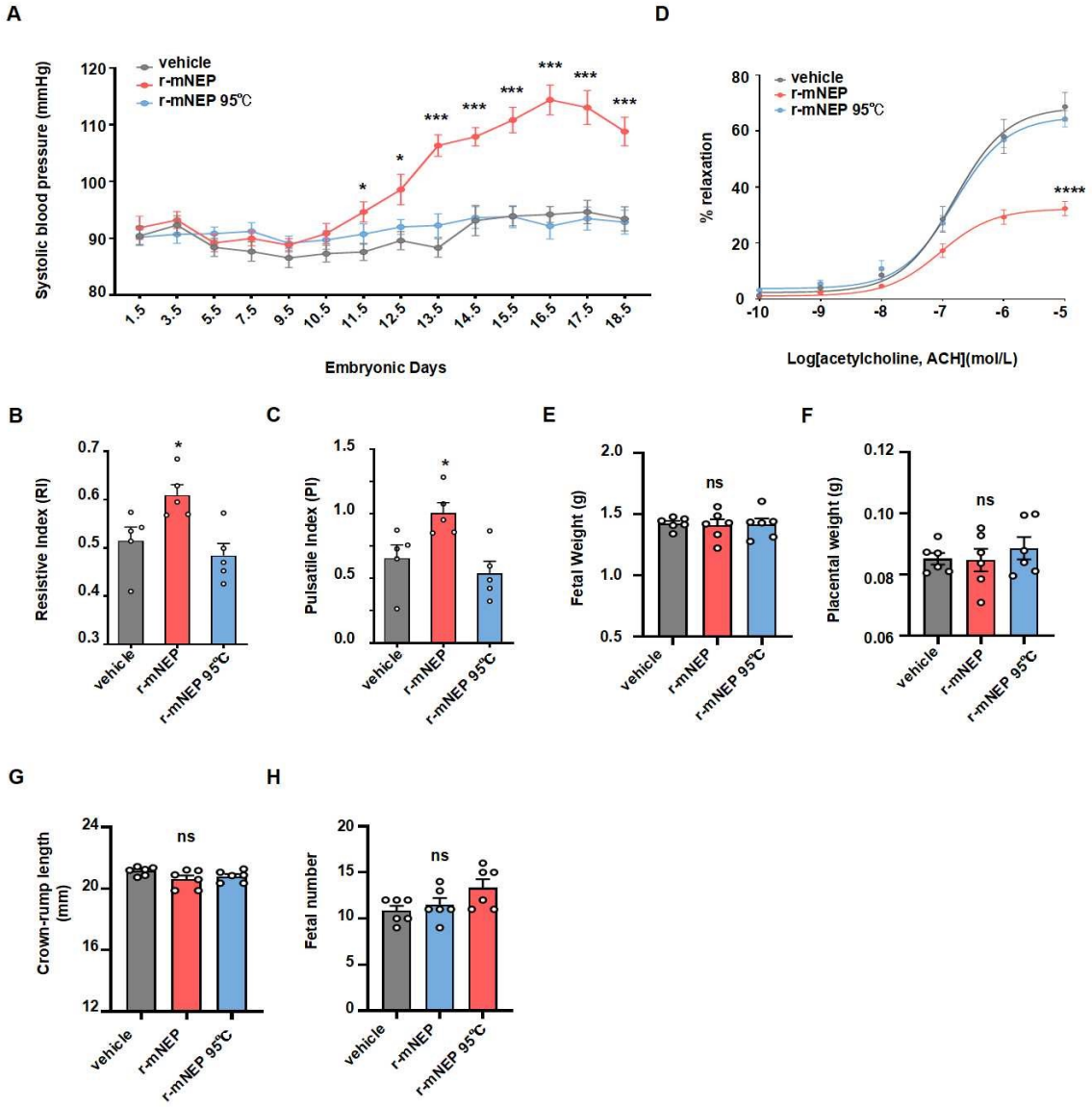

Figure 6. Injection of r-mNEP into pregnant mice impairs endothelium-dependent vasodilation of uterine arteries and leads to hypertension.

r-mNEP (400 ng in 200 μL) or the same volume of saline (vehicle) was intravenously infused into pregnant mice from E9.5 to E13.5. (A) Blood pressure measured by the tail-cuff method from E1.5 until E18.5 (n=8 in both vehicle and r-mNEP 95 °C group and n=10 in r-mNEP group). (B) The resistance index (RI) (n=5 dams) and (C) pulsatility index (PI)

in the uterine arteries were significantly higher in the mice infused with r-mNEP when analyzed by Doppler ultrasonography on E18.5 (n=5 dams). (D) The effect of r-mNEP on the ability of uterine arteries to undergo endothelium-dependent vasodilation in response to ACh was investigated by wire myography on E18.5 (n=8 dams). (E) Fetal BW, (F) placental weight, (G) CRL and (H) number of life fetuses on E18.5 (n=6 dams; ns, nonsignificant vs. vehicle and r-mNEP 95 °C). One-way ANOVA and Tukey's multiple range test were used in E-H; Two-way ANOVA and Tukey's multiple comparison test were used in A, D; The Kruskal-Wallis test and Dunn's multiple comparison were used in B-C. The results are presented as the means ± SEMs.

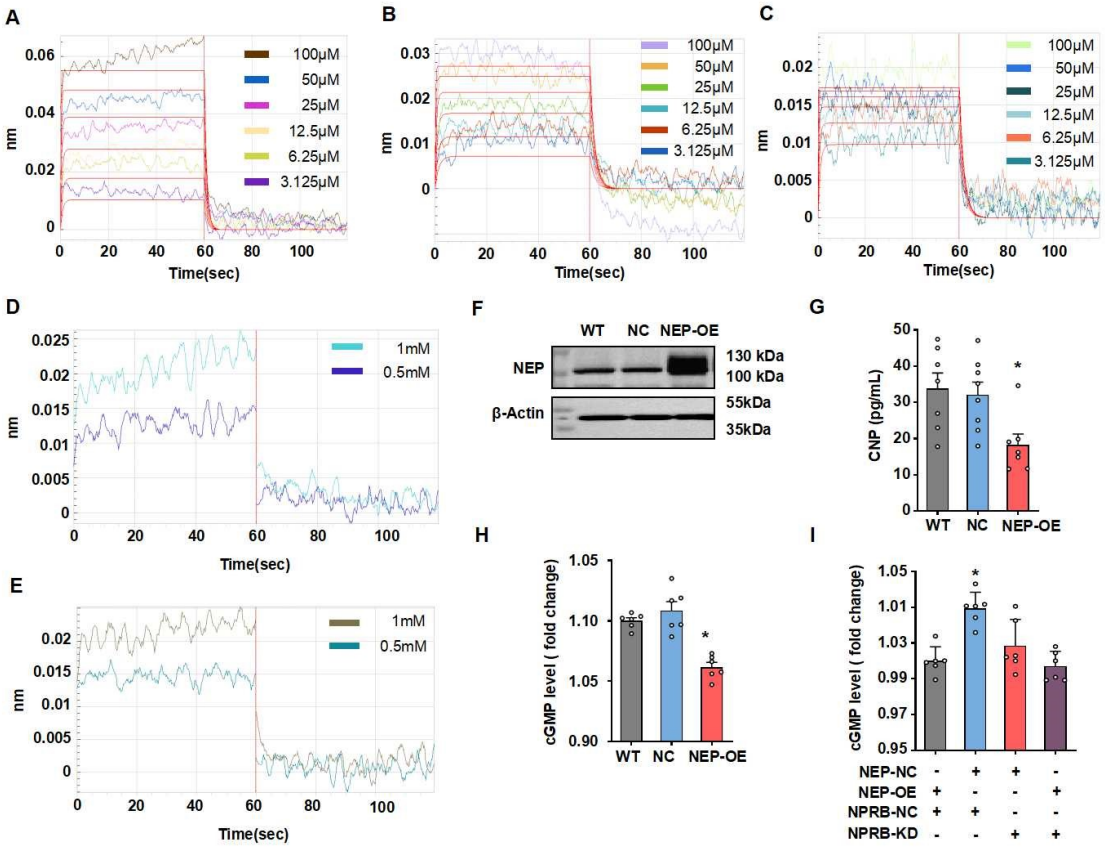

Figure 7. Elevated NEP in PL-EVs facilitates endothelial CNP degradation, which leads to a decrease in cGMP generated by CNP selectively binding to NPR-B.

Binding affinity measurements of NEP with (A) ANP, (B) ET-1, (C) CNP, (D) Ang II and (E) bradykinin assessed via the Forte Bio system. Curves correspond to the phases of association and dissociation of each peptide at various concentrations on the NEP anchored to the sensor chip. These curves were used to determine the K_D, Kon, and Koff. (F) NEP protein expression levels in NEP-overexpressing HUVECs were confirmed by Western blot analysis. β-Actin served as a loading control. (G) CNP levels in HUVEC culture medium were measured using a human CNP ELISA kit (n=7 samples in WT and NEP-OE groups and n=8 samples in NC group). (H) Fold changes in cGMP levels were measured

in WT A10 cells after coculture with WT, NC or NEP-OE HUVECs (n=6 samples). (I) Fold change in cGMP levels in A10 cells after coculture with HUVECs (n=6 samples). One-way ANOVA and Tukey's multiple range test were used in G, H-I. The results are presented as the means ± SEMs.

Novelty and Significance:

What is known?

- Preeclampsia (PE) is a kind of hypertensive disorder of pregnancy that seriously endangers the health of both the mother and the fetus, and there is still a lack of clinical treatment specifically for it.
- Placental Extracellular Vesicles (PL-EVs) from PE can transport substances generated from placenta to mother and some harmful molecules can cause serious diseases in the mother, such as hypertension and so on.
- Neprilysin (NEP) is a molecule that can lead to an increase in blood pressure, and its content has been found to be elevated in the PL-EVs of preeclampsia.

What New Information Does This Article Contribute?

- PL-EVs from PE pregnancies can impair endothelium-dependent vasodilation and induce PE-like symptoms in mice.
- Neprilysin (NEP), a PL-EV cargo protein mainly expressed by syncytiotrophoblasts, is upregulated in PE, and placenta-specific NEP-overexpressing mice show PE-like symptoms.
- NEP binds and degrades endothelial-released C-type natriuretic peptide (CNP), leading to insufficient CNP that compromises Natriuretic peptide receptor-B (NPR-B)-mediated cGMP production in vascular smooth muscle cells (VSMCs).
- Because PL-EVs regulate vascular tone via modulating inter-cellular communication between endothelial cells and VSMCs, the exploration of placental NEP and endothelial cell dysfunction is a promising interventional therapeutic target for PE.

Preeclampsia (PE), a hypertensive disorder of pregnancy severely endangering maternal and fetal health, still lacks specific clinical treatment. In this study, we found NEP, which is elevated in PEPL-EVs, is generated in syncytiotrophoblasts and transported to maternal endothelial cells and VSMCs, leading to the impairment of endothelium-dependent vasodilation. By treating pregnant mice with PEPL-EVs and placental-specific overexpressing ENP, mice can develop PE-like symptoms like hypertension, albuminuria and so on. Besides, we reveal the mechanism that NEP transported to endothelial cells can bind and degrade CNP, and decreased CNP may lead to the compromise of NPR-B-mediated cGMP production in vascular smooth muscle cells (VSMCs), ultimately resulting in impaired endothelium-dependent vasodilation and contributing to the development of PE-like symptoms. This study uncovers the novel role of NEP in preeclampsia pathogenesis and provides crucial theoretical basis for developing innovative therapeutic strategies against preeclampsia in clinic.