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# Acute Pressor Response to Psychosocial Stress Is Dependent on Endothelium-Derived Endothelin-1

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ORIGINAL RESEARCH

# Acute Pressor Response to Psychosocial Stress Is Dependent on Endothelium-Derived Endothelin-1

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***Background*—**Acute psychosocial stress provokes increases in circulating endothelin-1 (ET-1) levels in humans and animal models. However, key questions about the physiological function and cellular source of stress-induced ET-1 remain unanswered. We hypothesized that endothelium-derived ET-1 contributes to the acute pressor response to stress via activation of the endothelin A receptor.

***Methods and Results*—**Adult male vascular endothelium-specific ET-1 knockout mice and control mice that were homozygous for the floxed allele were exposed to acute psychosocial stress in the form of cage switch stress (CSS), with blood pressure measured by telemetry. An acute pressor response was elicited by CSS in both genotypes; however, this response was significantly blunted in vascular endothelium-specific ET-1 knockout mice compared with control mice that were homozygous for the floxed allele. In mice pretreated for 3 days with the endothelin A antagonist, ABT-627, or the dual endothelin A/B receptor antagonist, A-182086, the pressor response to CSS was similar between genotypes. CSS significantly increased plasma ET-1 levels in control mice that were homozygous for the floxed allele. CSS failed to elicit an increase in plasma ET-1 in vascular endothelium-specific ET-1 knockout mice. Telemetry frequency domain analyses suggested similar autonomic responses to stress between genotypes, and isolated resistance arteries demonstrated similar sensitivity to $\alpha_1$-adrenergic receptor-mediated vasoconstriction.

***Conclusions*—**These findings specify that acute stress-induced activation of endothelium-derived ET-1 and subsequent endothelin A receptor activation is a novel mediator of the blood pressure response to acute psychosocial stress. **(*J Am Heart Assoc*. 2018;7:e007863. DOI: 10.1161/JAHA.117.007863.)**

**Key Words:** cage switch stress • endothelin-1 • endothelium-derived factors • psychosocial stress • stress • vascular endothelium-specific ET-1 knockout mice



The acute experience of psychosocial stress induces a profound physiological reaction, leading to a rapid increase in blood pressure.[1–3] Acute stress responses are thought to prepare the body for action in life-threatening situations.[4] However, the modern human experience can repeatedly provoke the acute stress response in situations that are not necessarily life threatening, and abundant evidence now suggests that long-term exposure to psychosocial stress over time contributes to cardiovascular disease (CVD).[5,6] The largest study supporting this connection demonstrated that stress-provoking psychosocial factors have an independent association with acute myocardial infarction that is similar in magnitude to traditional cardiovascular risk factors.[7] Despite this, far less is known about the mechanisms through which stress-induced physiological processes contribute to the development of CVD.

Prior studies have identified endothelin-1 (ET-1) as a psychosocial stress-responsive factor that may represent a mechanistic mediator of the connection between stress and CVD.[8] ET-1 is a nanomolar potent peptide produced by diverse cell types throughout the body that functions in autocrine and paracrine signaling.[9,10] Multiple studies have demonstrated an increase in plasma ET-1 levels in response to laboratory-induced acute psychosocial stress in healthy, at risk, and diseased human subjects.[2,11–16] In population studies, psychosocial risk factors were associated with

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## Clinical Perspective

### What Is New?

- Endothelium-derived endothelin-1 signals to the endothelin A receptor contribute to the blood pressure response to acute psychosocial stress in mice.

### What Are the Clinical Implications?

- Psychosocial stress is associated with an increased risk of a wide range of cardiovascular complications and, consequently, understanding the mechanisms involved will inform future preventative and therapeutic strategies.
- We propose that exposure to psychosocial stressors leads to exaggerated activation of the endothelin-1/endothelin A pathway and that this phenomenon may represent a mechanistic link between psychosocial stress and cardiovascular disease risk.

elevated plasma ET-1 levels,[17,18] and acute psychosocial stress-induced acute coronary syndrome was associated with a robust elevation in plasma ET-1 levels.[19] In rodents, experimental acute psychosocial stress, in the form of restraint with air jet stress, leads to an increase in plasma ET-1 levels in both wild-type and prehypertensive rats.[20,21] Despite these studies, the cellular source of stress-induced plasma ET-1 remains unclear, because this ET-1 could be derived from the vascular endothelium, immune cells, neuronal cells, or other potential cellular sources. Interestingly, both acute and chronic stress hormones have been shown to increase ET-1 release in cultured endothelial cells.[22–24] Taken together, these studies demonstrate that a stress-mediated increase in plasma ET-1 is a consistent phenomenon that is conserved between humans and experimental animals.

We reasoned that basic questions about the physiological function and cellular source of stress-induced ET-1 were critical for understanding a future therapeutic role of the ET-1 pathway in stress paradigms. We hypothesized that psychosocial stress-induced ET-1 contributes to the rapid increase in blood pressure via activation of the endothelin A ($ET_A$) receptor and is derived from the endothelium. To test this hypothesis, we conducted experiments using vascular endothelium-specific ET-1 knockout mice (VEETKO) and control mice that were homozygous for the floxed allele (flox) male mice exposed to cage switch stress (CSS), an established experimental model of acute psychosocial stress.[3,25]

## Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

### Animals

All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and approved and monitored by the Medical College of Georgia at Augusta University or the University of Alabama at Birmingham Institutional Animal Care and Use Committees. VEETKO mice were developed previously using the Cre-lox system.[26] Knockout mice were additionally positive for the Tie2-Cre transgene (VEETKO). In total, 27 flox and 29 VEETKO mice were used for this study. Adult male mice, aged 6 months to 1 year, maintained on standard pellet chow diet (Harlan Teklad, Madison, WI) were used for all experiments. Mice were maintained on a 24-hour light-dark cycle, and experiments were conducted during the light period. For telemetry studies, mice were individually housed and provided with either normal drinking water, water containing the $ET_A$ receptor antagonist ABT-627 (atrasentan; 10 mg/kg per day; AbbVie, Inc, Abbott Park, IL),[27] or water containing the dual $ET_A$/endothelin B ($ET_B$) receptor antagonist A-182086 (10 mg/kg per day; AbbVie, Inc)[28] for 72 hours before the CSS experiment.

### Telemetry

Telemetry transmitters were implanted with catheters inserted into the right carotid artery of VEETKO and flox mice, as previously described.[29] Mice were allowed to recover for at least 10 days before experimentation. Telemetry recordings were initiated using DataQuest A.R.T. v.4.36 (Data Sciences International, Minneapolis, MN), and mice were left undisturbed for 12 hours before CSS, after which telemetry recordings were continued for 6 hours. Throughout this 18-hour period, telemetry recordings were obtained for the first 10 seconds of each minute at a sampling rate of 500 Hz. Telemetry files were imported into Ponemah v6.3 (DSI, Minneapolis, MN) for analysis of heart rate variability and frequency analysis of blood pressure variability. All segments were visualized manually, and periods of noise and artefactual triggering were excluded from further analysis. Beat-to-beat intervals were determined by triggering at maximum systolic pressure. Frequency cutoffs were set at low frequency (0.4–1.5 Hz) and high frequency (1.5–4.0 Hz).[30–32] Data were grouped into 30-minute bins immediately preceding and following the onset of CSS.

### CSS Protocol

The CSS protocol was developed to induce experimental psychosocial stress in male mice and has previously been used with a variety of mouse models.[3,25,33,34] Briefly, adult male flox or VEETKO mice, housed in groups of 2 or 3, were placed in a clean cage for 3 days before CSS stress. On the day of the CSS

experiment, at ≈1300 hours, 1 mouse per cage was randomly selected to represent baseline measurements and terminated for plasma and tissue collection, whereas a separate experimental mouse was exposed to CSS for 30 minutes by placing the mouse in a cage that previously housed male mice unknown to the experimental mouse for 3 days. Mice were then anesthetized (methohexital sodium, 40 mg/kg, IP), and cardiac puncture was performed using a 1-mL syringe rinsed with 7.5% EDTA. Blood samples were centrifuged at 1000*g* for 10 minutes at 4°C, and plasma was removed, aliquoted, and stored at −80°C. Adrenal glands were collected, snap frozen, and stored at −80°C. Of note, CSS elicits a pressor response for >60 minutes. Thus, we reasoned that 30 minutes would be necessary to observe an increase in plasma ET-1.

## Plasma ET-1

Plasma ET-1 peptide concentration was measured by ELISA (QuantiGlo; R&D Systems, Minneapolis, MN) in samples obtained from flox and VEETKO mice at baseline and following 30 minutes of CSS.

## *prepro-ET-1* mRNA

Relative levels of mRNA expression for *prepro-ET-1* were determined in thoracic aorta, renal vessels, and whole kidney, as previously reported.[21] Primers for the housekeeping gene, *Gapdh* (QT01658692), and *prepro-ET-1* (QT00253512) were purchased from Qiagen. Real-time quantitative reverse transcription–polymerase chain reaction was conducted using a Bio-Rad CFX96 Real-Time PCR System. Relative *prepro-ET-1* expression was calculated using $2^{-\Delta\Delta C}{}_{T}$ with the housekeeping gene *Gapdh*.

## ET-1/ET-3 Receptor Binding Assay

Radioligand ET-1 and ET-3 specific binding assays were performed using membrane-enriched fractions (300 ng of protein) from lung tissue, as previously described.[35] [$^{125}$I]ET-3 receptor binding was used to determine maximal binding values for $ET_B$ receptor number. To determine $ET_A$ receptor number, maximal binding values for [$^{125}$I]ET-3 binding were subtracted from [$^{125}$I]ET-1 maximal binding values representing total $ET_A$ and $ET_B$ receptor number.[35] Nonlinear regression analysis was conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA).

## Tissue Norepinephrine Content

Adrenal tissue was homogenized and centrifuged, and the supernatant was removed, as previously described.[36] The supernatant was extracted for quantification of norepinephrine levels by ELISA, according to manufacturer's instructions (Bi-CAT ELISA; Rocky Mountain Diagnostic, Inc).

## Vascular Reactivity

Thoracic aorta and third-order mesenteric artery segments were isolated and prepared for vascular reactivity experiments, as previously described.[37] Cumulative concentration-responses to phenylephrine were generated and normalized to the maximum constriction elicited by 100 mmol/L KCl.

## Statistical Analysis

All values are expressed as mean±SEM. Differences attributable to genotype and stress were compared using a 2-way ANOVA with Bonferroni post hoc test performed using GraphPad Prism version 7.0. Telemetry data were analyzed using 2-way repeated-measures ANOVA. Receptor binding assay data, mRNA expression data, baseline blood pressure data, and vascular reactivity data were analyzed using the 2-tailed, unpaired, Student *t* test. For all comparisons, $P<0.05$ was considered statistically significant.

# Results

## Vascular *prepro-ET-1* mRNA Expression Is Reduced in VEETKO Mice

To verify conditional knockout of ET-1 expression in the vascular endothelium, *prepro-ET-1* mRNA expression was assessed in isolated aorta, renal vessels, and whole kidney. Vascular *prepro-ET-1* mRNA levels were dramatically reduced in VEETKO mice compared with flox mice in both aorta (0.03±0.01 versus 1.00±0.23; $P=0.002$; flox, n=4; VEETKO, n=5) and renal vessels (0.37±0.12 versus 1.00±0.22; $P=0.030$; flox, n=4; VEETKO, n=5). Conversely, whole kidney *prepro-ET-1* mRNA level (0.94±0.34 versus 1.00±0.02; $P=0.856$; n=4 per group) was similar between genotypes, because kidneys are known to have high levels of ET-1 expression in the renal tubular epithelium.[38]

## Pressor Response to CSS Is Blunted in VEETKO Mice

We hypothesized that endothelium-derived ET-1 contributes to the blood pressure response to CSS. To test this, we monitored blood pressure, heart rate, and activity by telemetry in flox and VEETKO mice exposed to CSS. Baseline mean arterial pressure during the 3 hours before CSS did not differ between genotypes (flox versus VEETKO, 106.4±2.0 versus 100.2±2.7 mm Hg; $P=0.099$; flox, n=7; VEETKO, n=9). CSS induced a rapid increase in systolic, diastolic, and mean

arterial pressures in flox mice, with a maximum increase of ≈40 mm Hg, a magnitude consistent with previously published reports in control mice.[3,25] In contrast, the pressor response in the 10 minutes following CSS was significantly blunted in VEETKO mice (Figure 1A through 1C). The rapid increase in heart rate and locomotor activity induced by CSS did not differ between genotypes, suggesting that both flox and VEETKO mice reach a similar level of perceived acute psychosocial stress with the CSS protocol (Figure 1D and 1E).

## Specific $ET_A$ and Dual $ET_A$/$ET_B$ Receptor Antagonism Abolishes the Genotype Difference in the Pressor Response to CSS

The $ET_A$ receptor pathway is primarily responsible for the pressor response elicited by acute ET-1 infusion in mice.[39] Thus, we sought to determine if $ET_A$ receptor signaling is responsible for the contribution of endothelium-derived ET-1 to the stress-induced pressor response. To test this, telemeterized VEETKO and flox mice were pretreated with ABT-627 and subsequently exposed to CSS. Baseline mean arterial pressure in the 3 hours before CSS was similar between genotypes with ABT-627 treatment (flox versus VEETKO, 101.6±1.2 versus 101.3±2.4 mm Hg; *P*=0.904; flox, n=9; VEETKO, n=6). Short-term ABT-627 treatment abolished the genotype difference in the pressor response to CSS (Figure 2A through 2C). Similar to untreated mice, CSS-induced increases in heart rate and locomotor activity did not differ between ABT-627–treated flox and VEETKO mice (Figure 2D and 2E).

We further examined a potential role of $ET_B$ receptor signaling in the contribution of endothelium-derived ET-1 to the stress-induced pressor response. VEETKO and flox mice with telemetry units in place were pretreated with A-182086 and exposed to CSS. Baseline mean arterial pressure in the 3 hours before CSS was significantly lower in VEETKO compared with flox mice with A-182086 treatment

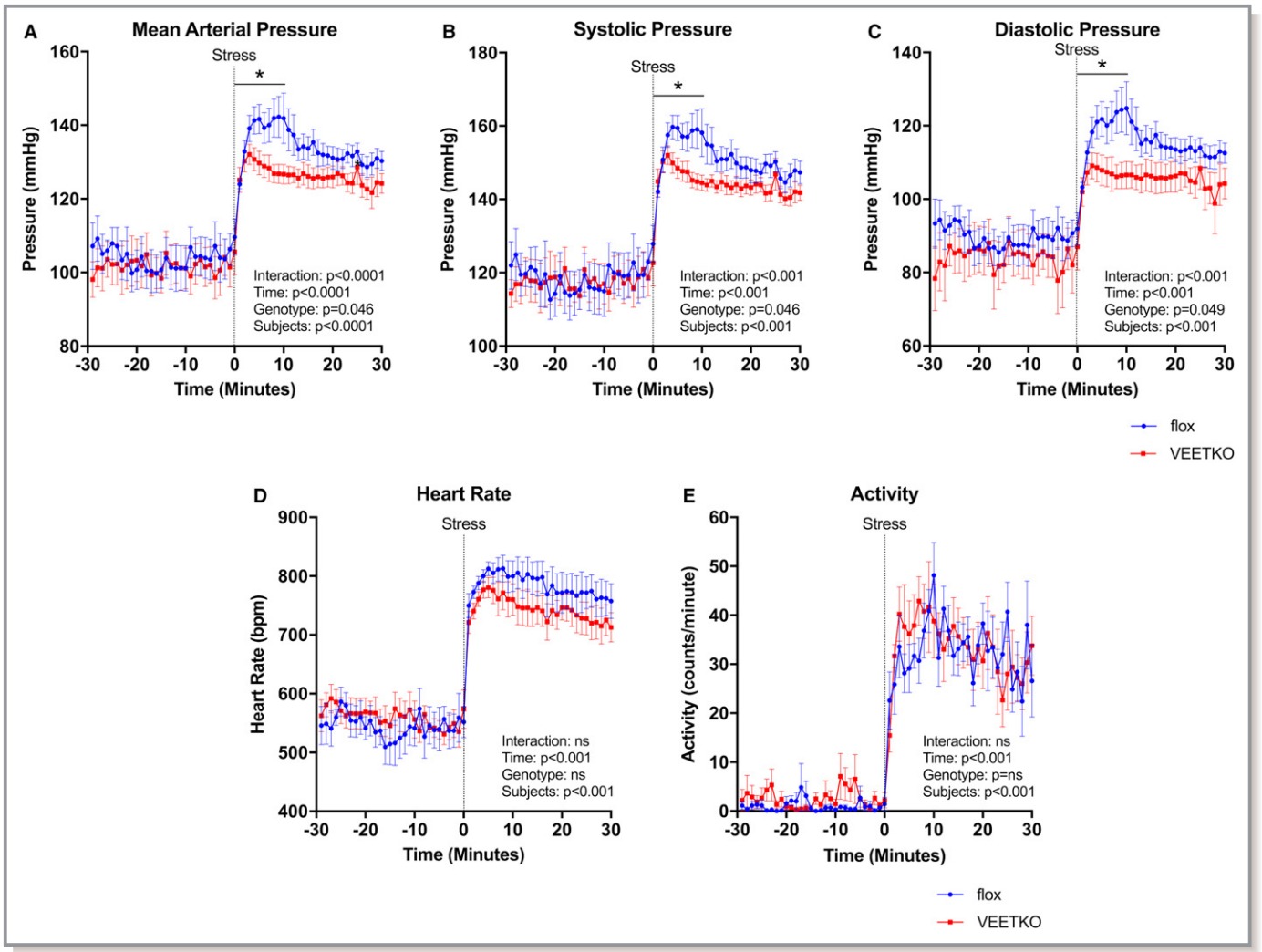


**Figure 1.** Blood pressure responses to cage switch stress (CSS) in vascular endothelium-specific endothelin-1 knockout (VEETKO) mice and control mice that were homozygous for the floxed allele (flox) receiving normal drinking water. Mean arterial pressure (A), systolic pressure (B), diastolic pressure (C), heart rate (D), and locomotor activity (E) in VEETKO (n=9) and flox (n=7) mice exposed to CSS.

(104.6±1.7 versus 110.7±1.4 mm Hg; *P*=0.015; flox, n=8; VEETKO, n=8). Similar to ABT-627 treatment, short-term A-182086 treatment abolished the genotype difference in the pressor response to CSS (Figure 3A through 3C). In contrast to vehicle and ABT-627 experiments, the heart rate response in the 10 minutes following CSS was significantly blunted in VEETKO mice pretreated with A-182086 (Figure 3D), whereas CSS-induced increases in locomotor activity were similar between genotypes (Figure 3E).

## CSS Failed to Increase Plasma ET-1 Levels in VEETKO Mice

To test the hypothesis that the endothelium is the source of stress-induced plasma ET-1, we exposed flox and VEETKO mice to CSS. Following 30 minutes of CSS exposure, plasma ET-1 levels were significantly elevated in flox mice (Figure 4A), consistent with observations in humans and rats exposed to psychosocial stress.[2,11–16,20,21] At baseline, plasma ET-1 levels were significantly lower in VEETKO mice compared with flox mice (Figure 4A). Furthermore, in VEETKO mice, CSS failed to induce a significant increase in plasma ET-1 levels (Figure 4A).

## Pulmonary $ET_B$ Receptor Activity Is Similar in Flox and VEETKO Mice

$ET_B$ receptors on the pulmonary endothelium represent the primary site of plasma ET-1 clearance.[10,40] Thus, we evaluated pulmonary ET receptor binding in VEETKO and flox mice. ET-1–specific binding was similar in VEETKO and flox mice (Figure 4C); in addition, ET-3–specific binding was similar across genotypes (Figure 4D). Calculated maximal binding of $ET_B$ receptor binding, and therefore $ET_B$ receptor expression,

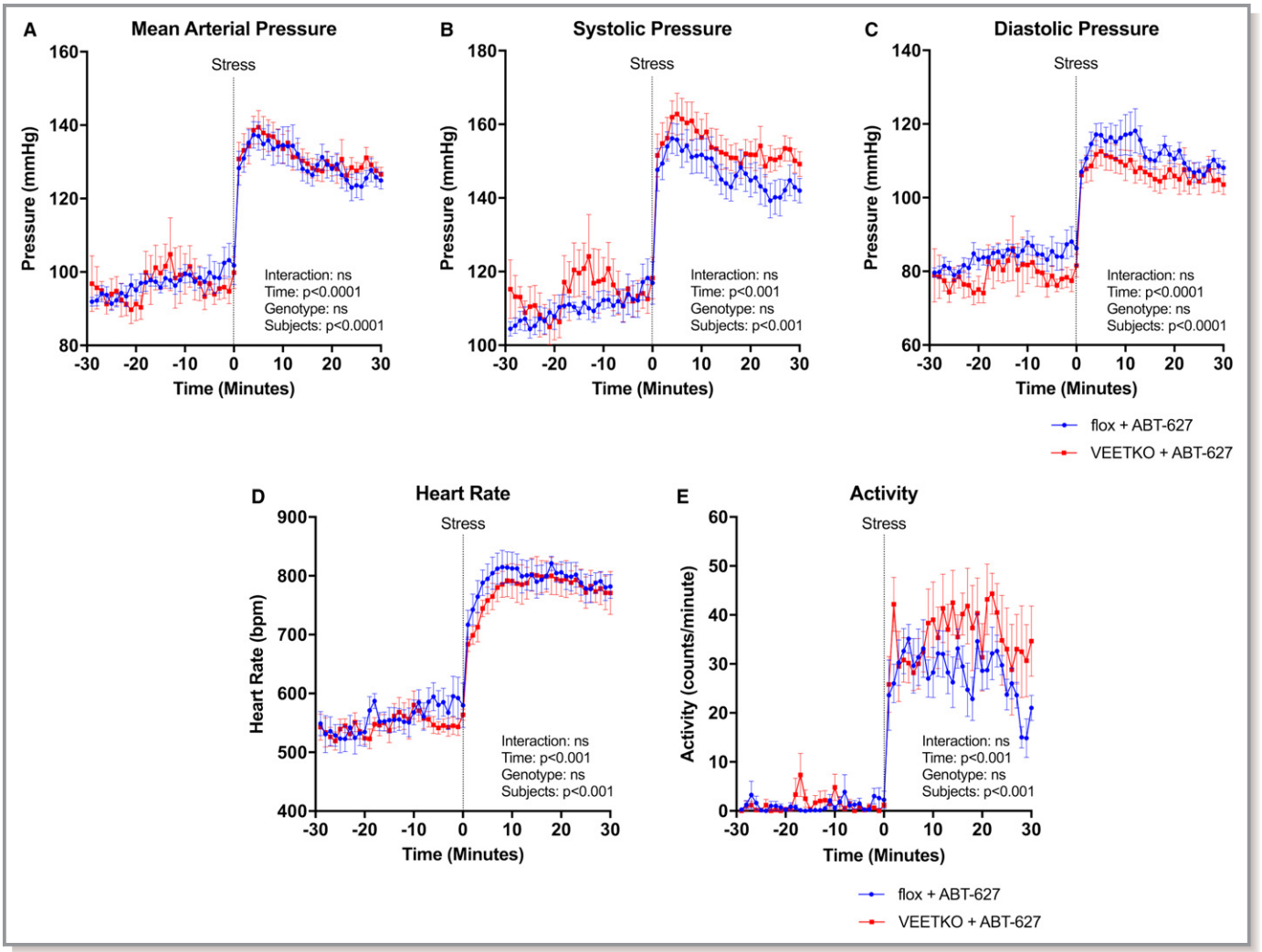


**Figure 2.** Blood pressure responses to cage switch stress (CSS) in vascular endothelium-specific endothelin-1 knockout (VEETKO) mice and control mice that were homozygous for the floxed allele (flox) receiving water containing ABT-627 for 3 days before CSS exposure. Mean arterial pressure (A), systolic pressure (B), diastolic pressure (C), heart rate (D), and locomotor activity (E) in VEETKO (n=6) and flox (n=9) mice.

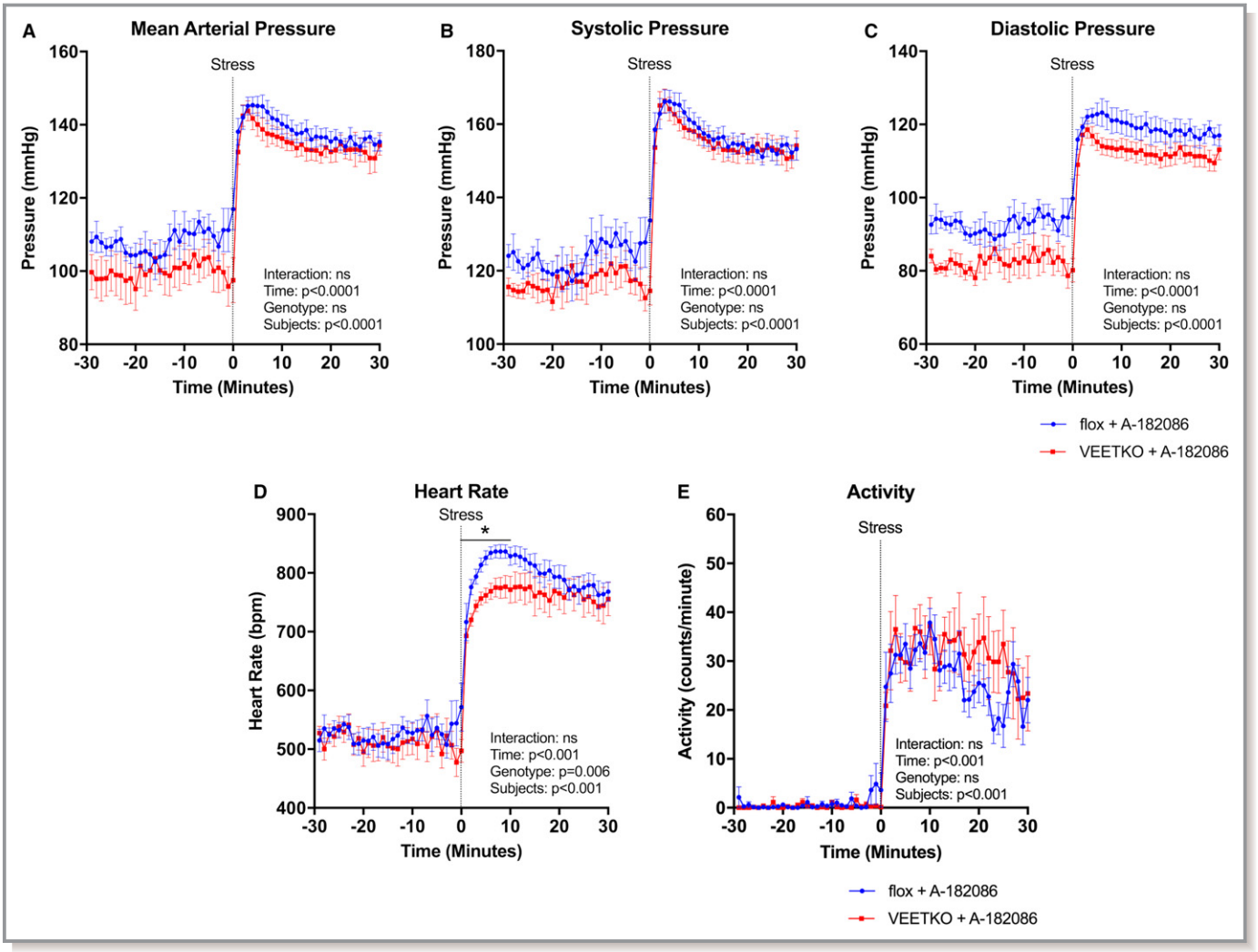


**Figure 3.** Blood pressure responses to cage switch stress (CSS) in vascular endothelium-specific endothelin-1 knockout (VEETKO) mice and control mice that were homozygous for the floxed allele (flox) receiving water containing A-182086 for 3 days before CSS exposure. Mean arterial pressure (A), systolic pressure (B), diastolic pressure (C), heart rate (D), and locomotor activity (E) in VEETKO (n=8) and flox (n=8) mice.

was similar in VEETKO and flox mice (Figure 4B). $ET_A$ receptor binding was not detected, as determined from the difference between ET-1 and ET-3 binding.

## Vasomotor Sympathetic/Parasympathetic Activation and Cardiac Parasympathetic Withdrawal in Response to CSS Are Similar in Flox and VEETKO Mice

Activation of the sympathetic nervous system and withdrawal of the parasympathetic nervous system occur in response to psychological stress.[41,42] Thus, we sought to compare autonomic responses to CSS in VEETKO and flox mice. Frequency domain analysis of systolic blood pressure variability low-/high-frequency ratio represents a general index of vasomotor sympathetic/parasympathetic activity. In the 3 hours before the onset of CSS, vehicle-treated VEETKO and flox mice demonstrated similar baseline systolic blood pressure variability low-/high-frequency ratio (1.3±0.2 versus 0.95±0.1; *P*=0.2; flox, n=7; VEETKO, n=9). In vehicle, ABT-627, and A-182086 treated VEETKO and flox mice, there was a significant effect of CSS to increase systolic blood pressure variability low-/high-frequency ratio but no significant effect of genotype (Figure 5A, 5C, and 5E). Analysis of heart rate variability, root means squared of the successive differences, represents a general index of cardiac parasympathetic activity. Baseline root means squared of the successive differences, in the 3 hours before the onset of CSS, was similar between genotypes (flox versus VEETKO, 5.6±0.7 versus 4.9±0.7 ms; *P*=0.5; flox, n=6; VEETKO, n=8). In vehicle, ABT-627, and A-182086 treated VEETKO and flox mice, there was a significant effect of CSS to decrease root means squared of the successive differences but no significant effect of genotype (Figure 5B, 5D, and 5F).

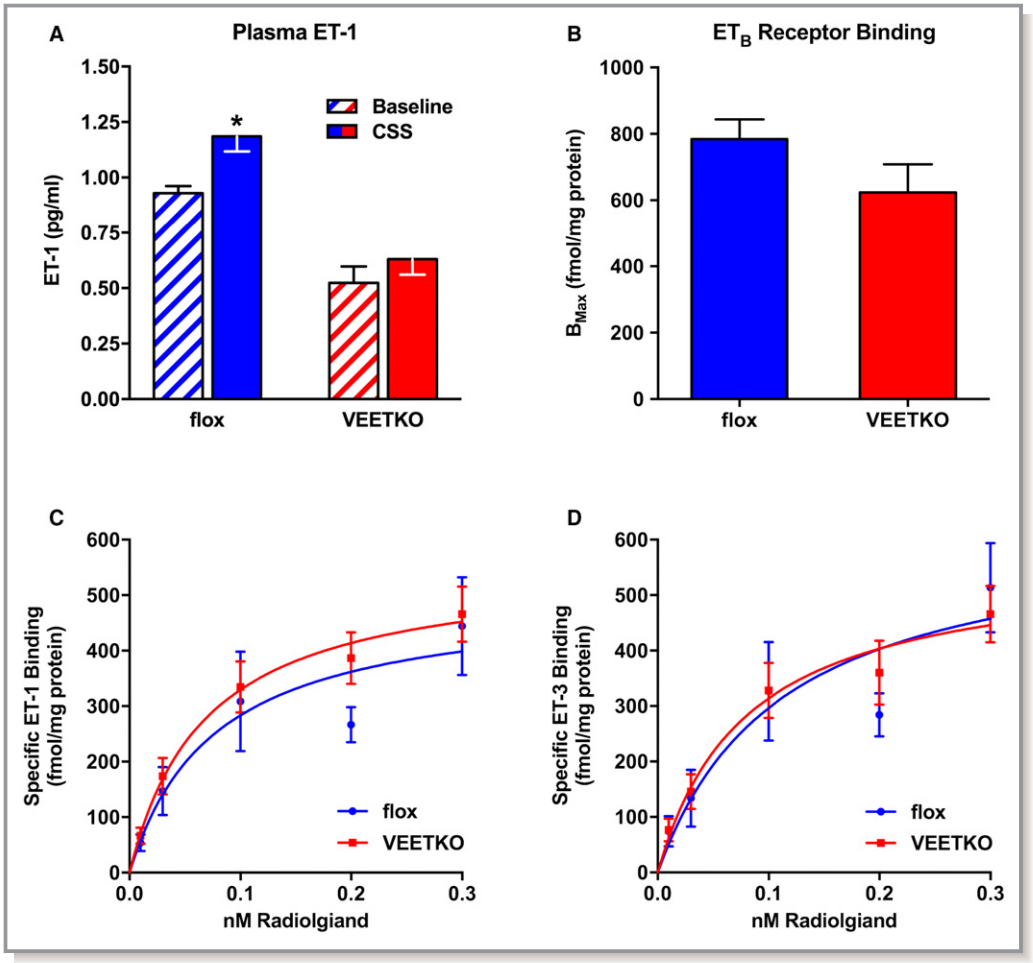


**Figure 4.** Plasma endothelin-1 (ET-1) peptide level in response to cage switch stress (CSS) and endothelin B ($ET_B$) receptor expression in lung membrane–enriched homogenates of vascular endothelium-specific ET-1 knockout (VEETKO) mice and control mice that were homozygous for the floxed allele (flox). A, Plasma ET-1 level is significantly elevated 30 minutes after the onset of CSS in flox control mice, but it is unchanged from baseline in VEETKO mice. *$P$<0.05 vs flox baseline; $P_{stress}$=0.014, $P_{genotype}$ <0.001, $P_{interaction}$=0.278; flox baseline (n=5), flox CSS (n=5), VEETKO baseline (n=6), VEETKO CSS (n=5); 2-way ANOVA with Bonferroni post hoc test. B, Calculated $ET_B$ receptor expression, $P$=0.174, 2-tailed, unpaired, Student $t$ test. C, ET-1–specific binding in flox and VEETKO mice (maximal binding [$B_{Max}$]: 580.8±112.6 vs 597.9±67.1 fmol/mg protein [$P$=0.901]; $K_d$: 0.11±0.03 vs 0.09±0.02 nmol/L [$P$=0.584]; 2-tailed, unpaired, Student $t$ test). D, ET-3 specific binding in flox (n=4) and VEETKO (n=4) mice ($B_{Max}$: 784.4±60.0 vs 623.7±84.9 fmol/mg protein [$P$=0.173]; $K_d$: 0.20±0.05 vs 0.11±0.03 nmol/L [$P$=0.166]; 2-tailed, unpaired, Student $t$ test). $K_d$ indicates dissociation constant.

## Adrenal Norepinephrine Levels Are Similarly Reduced in Flox and VEETKO Mice Following CSS

Adrenal norepinephrine release is a classic physiological component of the acute stress response. Therefore, to verify that the magnitude of stress experienced in response to CSS did not differ between genotypes, adrenal norepinephrine content was measured at baseline and following 30 minutes of CSS. Adrenal norepinephrine content was similar in flox and VEETKO mice at baseline, and following CSS, adrenal norepinephrine decreased to a similar level in the both genotypes (Figure 5G). Two-way ANOVA confirmed a significant effect of CSS to lower adrenal norepinephrine content but no significant effect of genotype (Figure 5H).

## Conduit and Resistance Artery $\alpha_1$-Adrenergic Sensitivity Is Similar in VEETKO and Flox Mice

To determine if a difference in arterial $\alpha_1$-adrenergic sensitivity may explain the genotype difference in the pressor response to CSS, phenylephrine responses were evaluated in isolated thoracic aorta and third-order mesenteric resistance arteries of VEEKO and flox mice. There were no significant genotype differences in the cumulative concentration

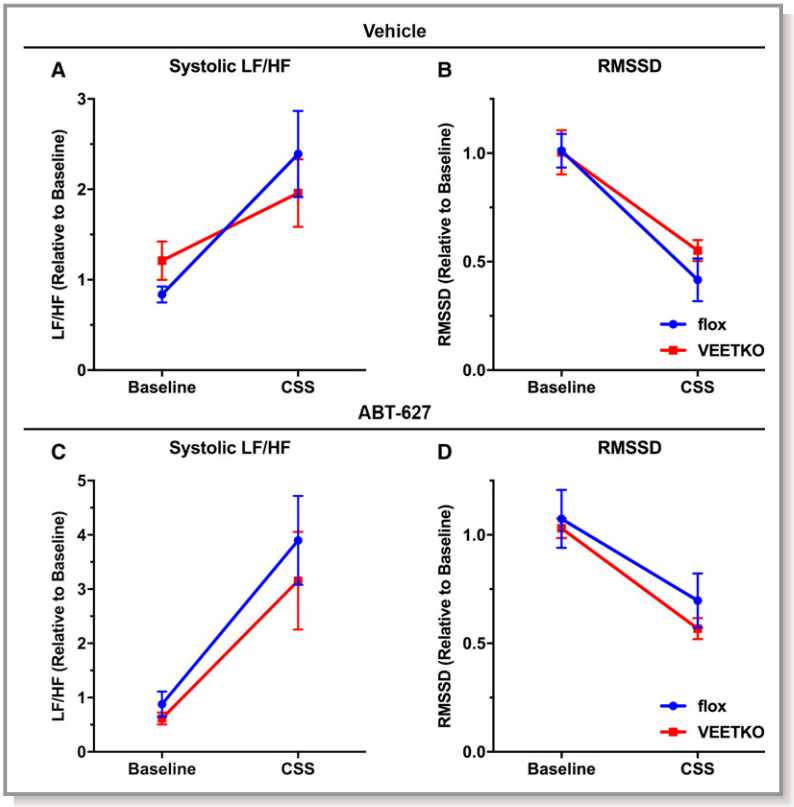


**Figure 5.** Heart rate variability and frequency blood pressure variability responses to cage switch stress (CSS) in vehicle, ABT-627, and A-182086 treated vascular endothelium-specific endothelin-1 knockout (VEETKO) mice and control mice that were homozygous for the floxed allele (flox). Systolic blood pressure variability low-/high-frequency ratio (Systolic LF/HF) in vehicle (n=7 flox, n=9 VEETKO; A), ABT-627 (n=8 flox, n=6 VEETKO; C), and A-182086 (n=8 flox, n=8 VEETKO; E) treated mice. Root means squared of the successive differences (RMSSD) relative to baseline in vehicle (n=6 flox, n=8 VEETKO; B), ABT-627 (n=8 flox, n=6 VEETKO; D), and A-182086 (n=8 flox, n=7 VEETKO; F) treated mice. G, Adrenal norepinephrine content at baseline and following 30 minutes of CSS exposure (n=4 flox baseline, n=4 flox CSS, n=6 VEETKO baseline, n=5 VEETKO CSS). H, Two-way repeated-measures ANOVA results (A through F) and ordinary 2-way ANOVA results (G).

response to phenylephrine in thoracic aorta or third-order mesenteric arteries (Figure 6A and 6B). In addition, the acute vasoconstriction response to $10^{-4}$ mol/L phenylephrine in thoracic aorta and third-order mesenteric arteries was similar between genotypes (Figure 6C and 6D).

## Discussion

Psychosocial stress is a robust independent risk factor for CVD,[7] yet there is limited understanding of the mechanisms underlying this association. ET-1 signaling has emerged as a candidate pathway from numerous studies in human and animal models that have demonstrated an increase in plasma ET-1 in response to acute psychosocial stress.[2,11–16,20,21] The major findings of the present study resolved important gaps in our understanding of this association. We identified the endothelium as an important source of acute stress-induced plasma ET-1 and determined that endothelium-derived ET-1 acts via $ET_A$ receptor activation to contribute to the acute stress-induced pressor response.

The pressor response to acute psychosocial stress is mediated by the coordinated action of multiple blood pressure regulatory systems, as determined by pharmacologic and genetic studies in mice and rats exposed to CSS. Specifically, adrenergic receptors play a critical role in mediating the pressor response to CSS, in which $\alpha_1$-adrenergic receptors contribute to the abrupt early increase in blood pressure and $\beta_1$-adrenergic receptors contribute to the sustained, but not immediate, response.[3] In the present study, we demonstrate that VEETKO mice exhibit blunting of the rapid increase in

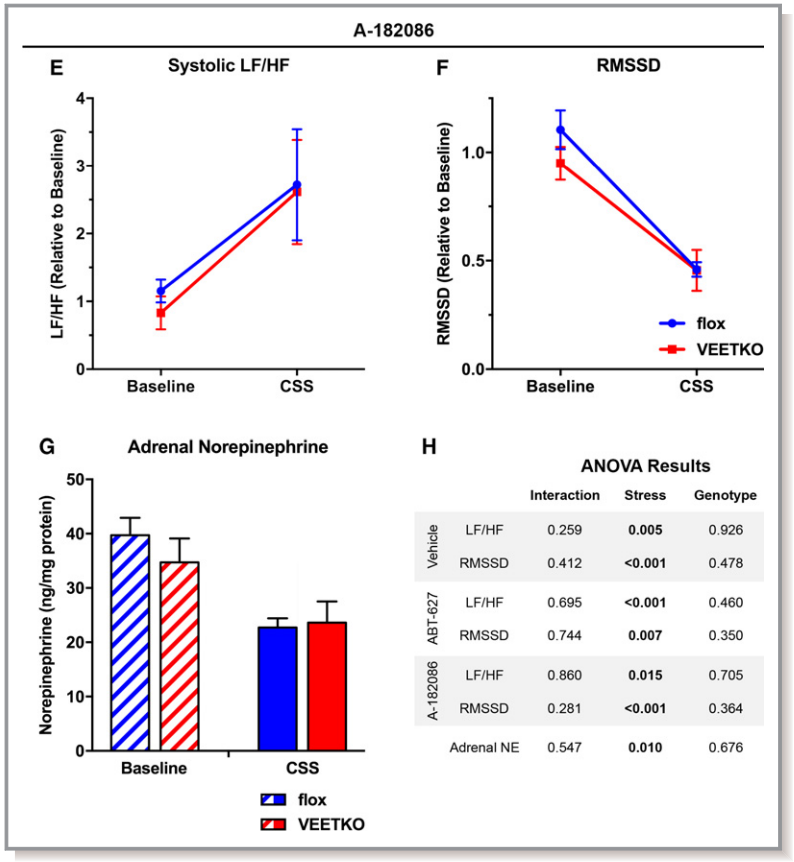


**Figure 5.** Continued

blood pressure in the first 10 minutes of the response to CSS and that the genotype difference in the pressor response diminishes with increasing time past the initiation of the stressor. These findings demonstrate that endothelium-derived ET-1 contributes to the early pressor response to CSS in mice, a contribution that is temporally similar to the contribution of $\alpha_1$-adrenergic receptors.[3] Therefore, we sought to determine if there is a difference in $\alpha_1$-adrenergic sensitivity in conduit and resistance arteries from flox and VEETKO mice. The results revealed that the vasoconstrictor response to the $\alpha_1$-adrenergic agonist, phenylephrine, was similar in VEETKO and flox mice, suggesting that enhancement of arterial $\alpha_1$-adrenergic sensitivity is not the mechanism by which endothelium-derived ET-1 contributes to the CSS-induced pressor response. These results are also consistent with the lack of a contribution of the ET pathway to the acute pressor response to phenylephrine infusion.[43,44] Further investigations have found that NO,[45] IL-6,[25] and corticotrophin-releasing hormone and activation of the hypothalamic-pituitary-adrenal axis[46] are directly involved in the physiological responses to CSS. The renin-angiotensin system plays a complex role in the pressor response to CSS, with $AT_{1A}$ receptors having both prohypertensive and antihypertensive roles, depending on their location.[3,33,34,47–49] Future research is needed to determine whether endothelium-derived ET-1 interacts with these other pathways or vice versa to mediate the acute pressor response. Taken together, these findings establish a role for endothelium-derived ET-1 in acute blood pressure regulation.

$ET_A$ receptor activation is primarily responsible for the vasoconstrictor actions of endogenous ET-1 and the hypertensive response to exogenous ET-1 infusion.[10,39] Herein, we demonstrate that 3-day pretreatment with the $ET_A$ antagonist, ABT-627, eliminates the genotype difference in the pressor response to CSS in VEETKO and flox mice. This finding suggests that endothelium-derived ET-1 acts on the $ET_A$ receptor to contribute to the pressor response to acute psychosocial stress. Our laboratory previously examined the contribution of the $ET_A$ receptor to the pressor response to restraint air jet stress in multiple strains of rats. D'Angelo et al found that ABT-627 potentiates the pressor response to air jet stress in $ET_B$ receptor–deficient rats and transgenic controls on a high-salt diet[50] and in Dahl salt-resistant, but not Dahl salt-sensitive, rats,[51] suggesting that the $ET_A$ receptor may function to attenuate sympathetic responses differentially in these rat strains. More important, the CSS paradigm is distinct from restraint air jet stress in that CSS elicits a prolonged pressor response, for >1 hour, and

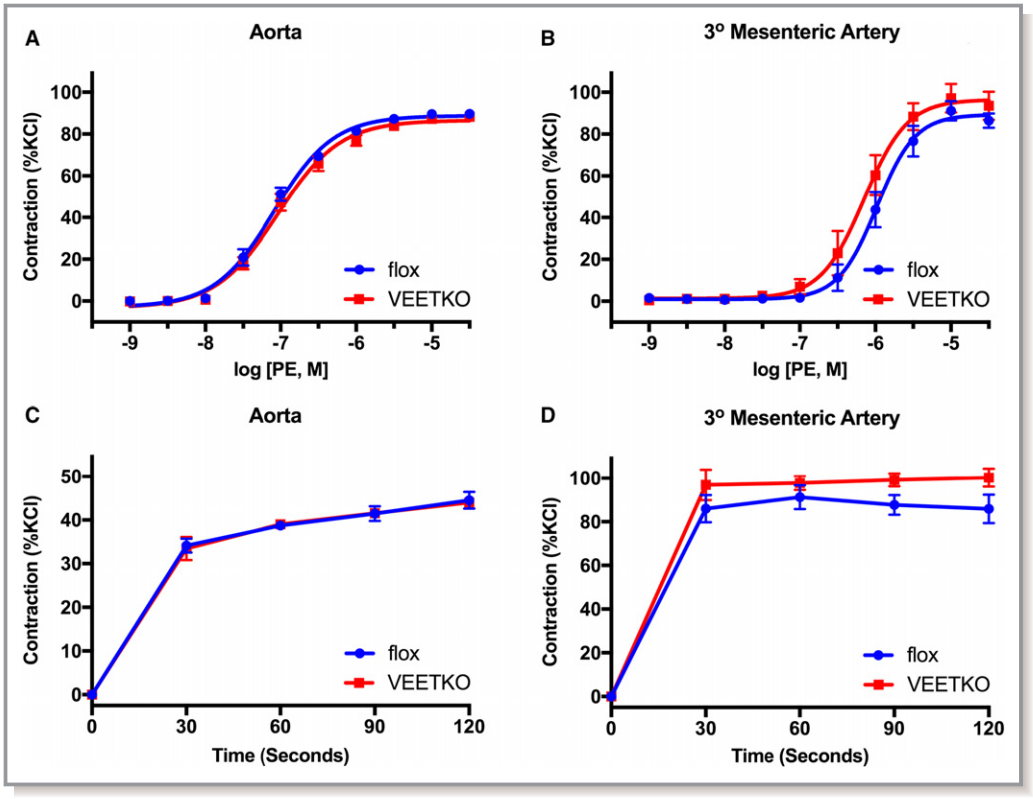


**Figure 6.** Vascular reactivity of isolated aorta and third-order mesenteric arteries of vascular endothelium-specific endothelin-1 knockout (VEETKO) mice and control mice that were homozygous for the floxed allele (flox). A, Cumulative concentration-response curves to phenylephrine (PE) in aorta of flox (n=4) and VEETKO (n=5) mice (Emax: 89.76±0.48% KCl vs 88.26±2.05% KCl [*P*=0.545]; $EC_{50}$: −7.089±0.062 vs −7.029±0.064 log [PE] [*P*=0.532]; 2-tailed, unpaired, Student *t* test). B, Cumulative concentration-response curves to PE in third-order mesenteric arteries of flox (n=4) and VEETKO (n=5) mice (Emax: 91.25±4.65% KCl vs 97.84±6.95% KCl [*P*=0.482]; $EC_{50}$: −5.982±0.108 vs −6.169±0.106 log [PE] [*P*=0.262]; 2-tailed, unpaired, Student *t* test). C, Acute constriction to $10^{-4}$ mol/L PE in aorta of flox (n=3) and VEETKO (n=5) mice (maximum contraction: 44.52±1.91% KCl and 44.01±1.05% KCl [*P*=0.805]; 2-tailed, unpaired, Student *t* test). D, Acute constriction to $10^{-4}$ mol/L PE in third-order mesenteric arteries of flox (n=4) and VEETKO (n=5) mice (maximum contraction: 95.54±5.60% KCl vs 103.58±3.50% KCl [*P*=0.244]; 2-tailed, unpaired, Student *t* test). Emax indicates maximal effective concentration.

restraint air jet stress produces a pressor response that dissipates in minutes.[21,46] Together, these studies suggest a complex role of the $ET_A$ receptor in the pressor response to acute psychosocial stress paradigms. Future studies are warranted using models of cell-specific conditional $ET_A$ deficiency exposed to acute psychosocial stressors.

The $ET_B$ receptor is expressed on sympathetic neurons, and recent studies in rats have demonstrated that exogenous activation of $ET_B$ receptors leads to an $\alpha_1$-adrenergic receptor-mediated pressor response.[52] Considering the importance of $\alpha_1$-adrenergic receptor activation to the CSS-induced pressor response and the herein identified role of endothelium-derived ET-1 in this response, we sought to determine if $ET_B$ receptor activation may contribute to the pressor response to CSS. $ET_B$ receptor antagonism alone results in hypertension that would confound pressor response results.[53] Thus, to test this, VEETKO and flox mice were pretreated for 3 days with the dual $ET_{A/B}$ receptor antagonist, A-182086. Similar to results with the $ET_A$ antagonist, A-182086 pretreatment eliminated the genotype difference in the pressor response to CSS, indicating no additional effect of $ET_B$ antagonism. This result suggests that endothelium-derived ET-1 acts on the $ET_A$ receptor to mediate its effect on the pressor response to CSS. Interestingly, A-182086 pretreatment led to a blunted heart rate response to CSS in VEETKO compared with flox mice, potentially revealing a component of the heart rate response to CSS that relies on the $ET_B$ receptor in a system lacking endothelium-derived ET-1.

Activation of the sympathetic nervous system and withdrawal of the parasympathetic nervous system occur in the short-term response to psychological stress.[41,42] Therefore, we sought to investigate the autonomic response to CSS. More important, baseline vasomotor sympathetic/parasympathetic activity and cardiac parasympathetic

activity were similar between genotypes. In response to CSS, there was an increase in vasomotor sympathetic/parasympathetic activity and a decrease in cardiac parasympathetic activity but no significant effect of genotype. In addition, there was an effect of CSS to reduce adrenal norepinephrine content but no significant effect of genotype, further suggesting similar sympathetic activation in VEETKO and flox mice. Together, these data suggest that differential autonomic responses do not underlie the contribution of endothelium-derived ET-1 to the pressor response to CSS. We speculate that sympathetic activation may be upstream of CSS-induced endothelium-derived ET-1, a concept that is consistent with the $\alpha_1$-adrenergic receptor–mediated ET-1 release that has been demonstrated in cultured endothelial cells.[22,24]

Consistent with prior studies in humans and rats,[2,11–16,20,21] flox mice exhibited a significant increase in plasma ET-1 in response to CSS, whereas VEETKO mice exhibited no change in plasma ET-1 following CSS. We found that pulmonary ET receptor expression and affinity are similar in VEETKO and flox mice, suggesting no differences in ET-1 clearance capacity in VEETKO and flox mice. Therefore, it is unlikely that the lack of significant induction of plasma ET-1 observed in VEETKO mice is the result of a genotype difference in ET-1 clearance capacity in these mice, although this is not directly tested. In addition, we provide multiple lines of evidence indicating that VEETKO and flox mice experienced an equivalent magnitude of experimental stress in response to CSS. We showed that both genotypes displayed a similar decrease in adrenal norepinephrine content, similar activation of vasomotor sympathetic/parasympathetic activity, and withdrawal of cardiac parasympathetic activity, as well as similar induction of locomotor activity in response to CSS. Taken together, these findings identify the endothelium as a major, but not necessarily exclusive, cellular source of ET-1 release in response to acute psychosocial stress. Our studies have not ruled out the possibility that other cell types, such as circulating immune cells, contribute to stress-induced plasma ET-1.

The present study demonstrated that endothelium-derived ET-1 contributes to the pressor response to acute psychosocial stress, and that this effect is dependent on the $ET_A$ receptor. Recent genome-wide association studies have implicated the $ET_A$ receptor as a genetic determinant of coronary artery disease,[54,55] carotid atherosclerosis,[54] large-artery stroke,[55] and peripheral artery disease,[56] suggesting a role of this receptor in atherogenesis across diverse arterial systems. These findings are supported by clinical and preclinical studies that suggest a direct role of the $ET_A$ receptor on the development of atherosclerosis.[57–61] Recently, Gupta and colleagues demonstrated in a separate genome-wide association study project that a single-nucleotide polymorphism common in 5 vascular diseases, including coronary artery disease, promotes exaggerated endothelium-derived ET-1 and higher plasma ET-1.[62] It would be interesting to determine whether $ET_A$ receptor and ET-1 single-nucleotide polymorphisms are coincident in vascular disease. Future studies are needed to determine if the psychosocial stress-mediated induction of endothelium-derived ET-1 may represent a mechanism connecting psychosocial stress to the development of CVD.

## Perspectives

Multiple studies in humans have observed an increase in plasma ET-1 in response to laboratory-induced stress,[11–16] and these findings have been further validated by a large clinical study of male and female participants.[2] More important, prior studies have highlighted the importance of both a family history of CVD and preexisting CVD as modifiers of the stress responsiveness of plasma ET-1, as reviewed by Yammine et al.[8] Thus, an increase in plasma ET-1 in response to stress is a physiological response that may be further amplified in the setting of vascular disease. In the setting of acute stress, endothelium-derived ET-1 release may lead to coronary vasoconstriction in patients with preexisting coronary artery disease. This effect may trigger or facilitate acute coronary syndrome, an idea consistent with the robustly elevated plasma ET-1 levels observed in stress-induced acute coronary syndrome.[19] Chronic psychosocial factors are associated with elevated plasma ET-1[17,18]; thus, long-term repeated exposure to psychosocial stressors may lead to persistently elevated endothelium-derived ET-1 release. Endothelium-specific ET-1 overexpressing mice display vascular oxidative stress and endothelial dysfunction,[63] alterations in genes related to lipid metabolism,[64] and increased vascular inflammation.[65] We propose that short- and long-term exposure to psychosocial stressors leads to exaggerated activation of the ET-1/$ET_A$ pathway and CVD risk.

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## Disclosures

None.

## References


1. McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev*. 2007;87:873–904.
2. Treiber FA, Kapuku GK, Davis H, Pollock JS, Pollock DM. Plasma endothelin-1 release during acute stress: role of ethnicity and sex. *Psychosom Med*. 2002;64:707–713.
3. Lee DL, Webb RC, Brands MW. Sympathetic and angiotensin-dependent hypertension during cage-switch stress in mice. *Am J Physiol Regul Integr Comp Physiol*. 2004;287:R1394–R1398.
4. Mobbs D, Hagan CC, Dalgleish T, Silston B, Prevost C. The ecology of human fear: survival optimization and the nervous system. *Front Neurosci*. 2015;9:55.
5. Dimsdale JE. Psychological stress and cardiovascular disease. *J Am Coll Cardiol*. 2008;51:1237–1246.
6. Seeman TE, McEwen BS, Rowe JW, Singer BH. Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. *Proc Natl Acad Sci USA*. 2001;98:4770–4775.
7. Rosengren A, Hawken S, Ounpuu S, Sliwa K, Zubaid M, Almahmeed WA, Blackett KN, Sitthi-amorn C, Sato H, Yusuf S; INTERHEART investigators. Association of psychosocial risk factors with risk of acute myocardial infarction in 11119 cases and 13648 controls from 52 countries (the INTERHEART study): case-control study. *Lancet*. 2004;364:953–962.
8. Yammine L, Kang DH, Baun MM, Meininger JC. Endothelin-1 and psychosocial risk factors for cardiovascular disease: a systematic review. *Psychosom Med*. 2014;76:109–121.
9. Kohan DE, Rossi NF, Inscho EW, Pollock DM. Regulation of blood pressure and salt homeostasis by endothelin. *Physiol Rev*. 2011;91:1–77.
10. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS, Pollock DM, Webb DJ, Maguire JJ. Endothelin. *Pharmacol Rev*. 2016;68:357–418.
11. Noll G, Wenzel RR, Schneider M, Oesch V, Binggeli C, Shaw S, Weidmann P, Luscher TF. Increased activation of sympathetic nervous system and endothelin by mental stress in normotensive offspring of hypertensive parents. *Circulation*. 1996;93:866–869.
12. Treiber FA, Jackson RW, Davis H, Pollock JS, Kapuku G, Mensah GA, Pollock DM. Racial differences in endothelin-1 at rest and in response to acute stress in adolescent males. *Hypertension*. 2000;35:722–725.
13. Mangiafico RA, Malatino LS, Attina T, Messina R, Fiore CE. Exaggerated endothelin release in response to acute mental stress in patients with intermittent claudication. *Angiology*. 2002;53:383–390.
14. Hartley B, Treiber F, Ludwig D, Kapuku G. Correlates of femoral artery flow mediated dilation in a multi-ethnic sample of 12- to 26-year-olds. *Ethn Dis*. 2004;14:227–232.
15. Fernandez AB, Soufer R, Collins D, Soufer A, Ranjbaran H, Burg MM. Tendency to angry rumination predicts stress-provoked endothelin-1 increase in patients with coronary artery disease. *Psychosom Med*. 2010;72:348–353.
16. Burg MM, Soufer A, Lampert R, Collins D, Soufer R. Autonomic contribution to endothelin-1 increase during laboratory anger-recall stress in patients with coronary artery disease. *Mol Med*. 2011;17:495–501.
17. Hong S, Nelesen RA, Krohn PL, Mills PJ, Dimsdale JE. The association of social status and blood pressure with markers of vascular inflammation. *Psychosom Med*. 2006;68:517–523.
18. Cooper DC, Mills PJ, Bardwell WA, Ziegler MG, Dimsdale JE. The effects of ethnic discrimination and socioeconomic status on endothelin-1 among blacks and whites. *Am J Hypertens*. 2009;22:698–704.
19. Wilbert-Lampen U, Nickel T, Leistner D, Guthlin D, Matis T, Volker C, Sper S, Kuchenhoff H, Kaab S, Steinbeck G. Modified serum profiles of inflammatory and vasoconstrictive factors in patients with emotional stress-induced acute coronary syndrome during World Cup soccer 2006. *J Am Coll Cardiol*. 2010;55:637–642.
20. D'Angelo G, Loria AS, Pollock DM, Pollock JS. Endothelin activation of reactive oxygen species mediates stress-induced pressor response in Dahl salt-sensitive prehypertensive rats. *Hypertension*. 2010;56:282–289.
21. Loria AS, D'Angelo G, Pollock DM, Pollock JS. Early life stress downregulates endothelin receptor expression and enhances acute stress-mediated blood pressure responses in adult rats. *Am J Physiol Regul Integr Comp Physiol*. 2010;299:R185–R191.
22. Prasad MR, Jones RM, Kreutzer DL. Release of endothelin from cultured bovine endothelial cells. *J Mol Cell Cardiol*. 1991;23:655–658.
23. Nickel T, Deutschmann A, Hanssen H, Summo C, Wilbert-Lampen U. Modification of endothelial biology by acute and chronic stress hormones. *Microvasc Res*. 2009;78:364–369.
24. Kohno M, Murakawa K, Yokokawa K, Yasunari K, Horio T, Kurihara N, Takeda T. Production of endothelin by cultured porcine endothelial cells: modulation by adrenaline. *J Hypertens Suppl*. 1989;7:S130–S131.
25. Lee DL, Leite R, Fleming C, Pollock JS, Webb RC, Brands MW. Hypertensive response to acute stress is attenuated in interleukin-6 knockout mice. *Hypertension*. 2004;44:259–263.
26. Kisanuki YY, Emoto N, Ohuchi T, Widyantoro B, Yagi K, Nakayama K, Kedzierski RM, Hammer RE, Yanagisawa H, Williams SC, Richardson JA, Suzuki T, Yanagisawa M. Low blood pressure in endothelial cell-specific endothelin 1 knockout mice. *Hypertension*. 2010;56:121–128.
27. Speed JS, Heimlich JB, Hyndman KA, Fox BM, Patel V, Yanagisawa M, Pollock JS, Titze JM, Pollock DM. Endothelin-1 as a master regulator of whole-body Na+ homeostasis. *FASEB J*. 2015;29:4937–4944.
28. Kasztan M, Fox BM, Speed JS, De Miguel C, Gohar EY, Townes TM, Kutlar A, Pollock JS, Pollock DM. Long-term endothelin-a receptor antagonism provides robust renal protection in humanized sickle cell disease mice. *J Am Soc Nephrol*. 2017;28:2443–2458.
29. Hyndman KA, Boesen EI, Elmarakby AA, Brands MW, Huang P, Kohan DE, Pollock DM, Pollock JS. Renal collecting duct NOS1 maintains fluid-electrolyte homeostasis and blood pressure. *Hypertension*. 2013;62:91–98.
30. Duan SZ, Christe M, Milstone DS, Mortensen RM. Go but not Gi2 or Gi3 is required for muscarinic regulation of heart rate and heart rate variability in mice. *Biochem Biophys Res Commun*. 2007;357:139–143.
31. Williams TD, Chambers JB, Gagnon SP, Roberts LM, Henderson RP, Overton JM. Cardiovascular and metabolic responses to fasting and thermoneutrality in ay mice. *Physiol Behav*. 2003;78:615–623.
32. Thireau J, Zhang BL, Poisson D, Babuty D. Heart rate variability in mice: a theoretical and practical guide. *Exp Physiol*. 2008;93:83–94.
33. Davern PJ, Chen D, Head GA, Chavez CA, Walther T, Mayorov DN. Role of angiotensin II type 1A receptors in cardiovascular reactivity and neuronal activation after aversive stress in mice. *Hypertension*. 2009;54:1262–1268.
34. Chen D, Jancovski N, Bassi JK, Nguyen-Huu TP, Choong YT, Palma-Rigo K, Davern PJ, Gurley SB, Thomas WG, Head GA, Allen AM. Angiotensin type 1A receptors in C1 neurons of the rostral ventrolateral medulla modulate the pressor response to aversive stress. *J Neurosci*. 2012;32:2051–2061.
35. Taylor TA, Gariepy CE, Pollock DM, Pollock JS. Unique endothelin receptor binding in kidneys of ETB receptor deficient rats. *Am J Physiol Regul Integr Comp Physiol*. 2003;284:R674–R681.
36. Loria AS, Brands MW, Pollock DM, Pollock JS. Early life stress sensitizes the renal and systemic sympathetic system in rats. *Am J Physiol Renal Physiol*. 2013;305:F390–F395.
37. Kang KT, Sullivan JC, Sasser JM, Imig JD, Pollock JS. Novel nitric oxide synthase–dependent mechanism of vasorelaxation in small arteries from hypertensive rats. *Hypertension*. 2007;49:893–901.
38. Speed JS, Fox BM, Johnston JG, Pollock DM. Endothelin and renal ion and water transport. *Semin Nephrol*. 2015;35:137–144.
39. Donato AJ, Lesniewski LA, Stuart D, Walker AE, Henson G, Sorensen L, Li D, Kohan DE. Smooth muscle specific disruption of the endothelin-a receptor in mice reduces arterial pressure, and vascular reactivity and affects vascular development. *Life Sci*. 2014;118:238–243.
40. Fukuroda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M, Nishikibe M. Clearance of circulating endothelin-1 by ETB receptors in rats. *Biochem Biophys Res Commun*. 1994;199:1461–1465.
41. Berntson GG, Cacioppo JT, Binkley PF, Uchino BN, Quigley KS, Fieldstone A. Autonomic cardiac control, III: psychological stress and cardiac response in autonomic space as revealed by pharmacological blockades. *Psychophysiology*. 1994;31:599–608.
42. Inagaki H, Kuwahara M, Tsubone H. Effects of psychological stress on autonomic control of heart in rats. *Exp Anim*. 2004;53:373–378.
43. Richard V, Hogie M, Clozel M, Loffler BM, Thuillez C. In vivo evidence of an endothelin-induced vasopressor tone after inhibition of nitric oxide synthesis in rats. *Circulation*. 1995;91:771–775.
44. Ballew JR, Watts SW, Fink GD. Effects of salt intake and angiotensin II on vascular reactivity to endothelin-1. *J Pharmacol Exp Ther*. 2001;296:345–350.

45. Uji M, Yoshida K, Shintani-Ishida K, Morimoto K. Sex difference in norepinephrine surge in response to psychological stress through nitric oxide in rats. *Life Sci*. 2007;80:860–866.
46. Morimoto A, Nakamori T, Morimoto K, Tan N, Murakami N. The central role of corticotrophin-releasing factor (CRF-41) in psychological stress in rats. *J Physiol*. 1993;460:221–229.
47. Abegaz B, Davern PJ, Jackson KL, Nguyen-Huu TP, Bassi JK, Connelly A, Choong YT, Allen AM, Head GA. Cardiovascular role of angiotensin type1A receptors in the nucleus of the solitary tract of mice. *Cardiovasc Res*. 2013;100:181–191.
48. Palma-Rigo K, Bassi JK, Nguyen-Huu TP, Jackson KL, Davern PJ, Chen D, Elghozi JL, Thomas WG, Allen AM, Head GA. Angiotensin 1A receptors transfected into caudal ventrolateral medulla inhibit baroreflex gain and stress responses. *Cardiovasc Res*. 2012;96:330–339.
49. Oscar CG, Muller-Ribeiro FC, de Castro LG, Martins Lima A, Campagnole-Santos MJ, Santos RA, Xavier CH, Fontes MA. Angiotensin-(1-7) in the basolateral amygdala attenuates the cardiovascular response evoked by acute emotional stress. *Brain Res*. 2015;1594:183–189.
50. D'Angelo G, Pollock JS, Pollock DM. Endogenous endothelin attenuates the pressor response to acute environmental stress via the eta receptor. *Am J Physiol Heart Circ Physiol*. 2005;288:H1829–H1835.
51. D'Angelo G, Pollock JS, Pollock DM. In vivo evidence for endothelin-1-mediated attenuation of alpha1-adrenergic stimulation. *Am J Physiol Heart Circ Physiol*. 2006;290:H1251–H1258.
52. Becker BK, Speed JS, Powell M, Pollock DM. Activation of neuronal endothelin B receptors mediates pressor response through alpha-1 adrenergic receptors. *Physiol Rep*. 2017;5:e13077.
53. Ohuchi T, Kuwaki T, Ling GY, Dewit D, Ju KH, Onodera M, Cao WH, Yanagisawa M, Kumada M. Elevation of blood pressure by genetic and pharmacological disruption of the ETB receptor in mice. *Am J Physiol*. 1999;276:R1071–R1077.
54. Bis JC, Kavousi M, Franceschini N, Isaacs A, Abecasis GR, Schminke U, Post WS, Smith AV, Cupples LA, Markus HS, Schmidt R, Huffman JE, Lehtimaki T, Baumert J, Munzel T, Heckbert SR, Dehghan A, North K, Oostra B, Bevan S, Stoegerer EM, Hayward C, Raitakari O, Meisinger C, Schillert A, Sanna S, Volzke H, Cheng YC, Thorsson B, Fox CS, Rice K, Rivadeneira F, Nambi V, Halperin E, Petrovic KE, Peltonen L, Wichmann HE, Schnabel RB, Dorr M, Parsa A, Aspelund T, Demissie S, Kathiresan S, Reilly MP, Taylor K, Uitterlinden A, Couper DJ, Sitzer M, Kahonen M, Illig T, Wild PS, Orru M, Ludemann J, Shuldiner AR, Eiriksdottir G, White CC, Rotter JI, Hofman A, Seissler J, Zeller T, Usala G, Ernst F, Launer LJ, D'Agostino RB Sr, O'Leary DH, Ballantyne C, Thiery J, Ziegler A, Lakatta EG, Chilukoti RK, Harris TB, Wolf PA, Psaty BM, Polak JF, Li X, Rathmann W, Uda M, Boerwinkle E, Klopp N, Schmidt H, Wilson JF, Viikari J, Koenig W, Blankenberg S, Newman AB, Witteman J, Heiss G, Duijn C, Scuteri A, Homuth G, Mitchell BD, Gudnason V, O'Donnell CJ; CARDIoGRAM Consortium. Meta-analysis of genome-wide association studies from the CHARGE consortium identifies common variants associated with carotid intima media thickness and plaque. *Nat Genet*. 2011;43:940–947.
55. Dichgans M, Malik R, Konig IR, Rosand J, Clarke R, Gretarsdottir S, Thorleifsson G, Mitchell BD, Assimes TL, Levi C, O'Donnell CJ, Fornage M, Thorsteinsdottir U, Psaty BM, Hengstenberg C, Seshadri S, Erdmann J, Bis JC, Peters A, Boncoraglio GB, Marz W, Meschia JF, Kathiresan S, Ikram MA, McPherson R, Stefansson K, Sudlow C, Reilly MP, Thompson JR, Sharma P, Hopewell JC, Chambers JC, Watkins H, Rothwell PM, Roberts R, Markus HS, Samani NJ, Farrall M, Schunkert H; METASTROKE Consortium, CARDIoGRAM Consortium, C4D Consortium, International Stroke Genetics Consortium. Shared genetic susceptibility to ischemic stroke and coronary artery disease: a genome-wide analysis of common variants. *Stroke*. 2014;45:24–36.
56. Matsukura M, Ozaki K, Takahashi A, Onouchi Y, Morizono T, Komai H, Shigematsu H, Kudo T, Inoue Y, Kimura H, Hosaka A, Shigematsu K, Miyata T, Watanabe T, Tsunoda T, Kubo M, Tanaka T. Genome-wide association study of peripheral arterial disease in a Japanese population. *PLoS One*. 2015;10:e0139262.
57. Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Munter K, Luscher TF. Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc Natl Acad Sci U S A*. 1998;95:14367–14372.
58. Iwasa S, Fan J, Miyauchi T, Watanabe T. Blockade of endothelin receptors reduces diet-induced hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice. *Pathobiology*. 2001;69:1–10.
59. Babaei S, Picard P, Ravandi A, Monge JC, Lee TC, Cernacek P, Stewart DJ. Blockade of endothelin receptors markedly reduces atherosclerosis in LDL receptor deficient mice: role of endothelin in macrophage foam cell formation. *Cardiovasc Res*. 2000;48:158–167.
60. Reriani M, Raichlin E, Prasad A, Mathew V, Pumper GM, Nelson RE, Lennon R, Rihal C, Lerman LO, Lerman A. Long-term administration of endothelin receptor antagonist improves coronary endothelial function in patients with early atherosclerosis. *Circulation*. 2010;122:958–966.
61. Yoon MH, Reriani M, Mario G, Rihal C, Gulati R, Lennon R, Tilford JM, Lerman LO, Lerman A. Long-term endothelin receptor antagonism attenuates coronary plaque progression in patients with early atherosclerosis. *Int J Cardiol*. 2013;168:1316–1321.
62. Gupta RM, Hadaya J, Trehan A, Zekavat SM, Roselli C, Klarin D, Emdin CA, Hilvering CRE, Bianchi V, Mueller C, Khera AV, Ryan RJH, Engreitz JM, Issner R, Shoresh N, Epstein CB, de Laat W, Brown JD, Schnabel RB, Bernstein BE, Kathiresan S. A genetic variant associated with five vascular diseases is a distal regulator of endothelin-1 gene expression. *Cell*. 2017;170:522–533.e515
63. Amiri F, Virdis A, Neves MF, Iglarz M, Seidah NG, Touyz RM, Reudelhuber TL, Schiffrin EL. Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation*. 2004;110:2233–2240.
64. Simeone SM, Li MW, Paradis P, Schiffrin EL. Vascular gene expression in mice overexpressing human endothelin-1 targeted to the endothelium. *Physiol Genomics*. 2011;43:148–160.
65. Li MW, Mian MO, Barhoumi T, Rehman A, Mann K, Paradis P, Schiffrin EL. Endothelin-1 overexpression exacerbates atherosclerosis and induces aortic aneurysms in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol*. 2013;33:2306–2315.