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Control of blood pressure variability in caveolin-1-deficient mice: role of nitric oxide identified *in vivo* through spectral analysis

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Aims In endothelial cells, caveolin-1 (cav-1) is known to negatively modulate the activation of endothelial nitric oxide synthase, a key regulator of blood pressure (BP). However, the impact of genetic alteration of cav-1 on vascular nitric oxide (NO) production and BP homeostasis *in vivo* is unknown.
Methods and results We used spectral analysis of systolic blood pressure (SBP) variability in mice chronically equipped with telemetry implants to identify frequency ranges (0.05–0.4 Hz; very low frequency, VLF) specifically responding to NO, independently of changes in absolute BP or systemic neurohormone levels. VLF variability was inversely correlated to aortic vasodilator-stimulated Ser^{239} phosphoprotein (VASP) phosphorylation, reflecting NO bioactivity. We show that mice deficient in cav-1 have decreased VLF variability paralleled with enhanced systemic and vascular production of NO at unchanged mean SBP levels. Conversely, VLF variability was increased upon acute injection of mice, with a peptide containing the caveolin-scaffolding domain (CSD; residues 82–101) fused to an internalization sequence of antennapedia that decreased vascular and circulating NO *in vivo*.
Conclusion These data highlight the functional importance of cav-1 for the production of bioactive NO in conduit arteries and its control of central BP variability. Given the impact of the latter on target organ damage, this raises the interest for genetic, pharmacological, or molecular interventions that modulate cav-1 expression in diseases with NO-dependent endothelial dysfunction.

1. Introduction

Among the variety of paracrine factors released by the endothelium, nitric oxide (NO) has been recognized for the last 20 years as a key regulator of the biology and contraction of the vascular wall, as well as the rheology of circulating blood cells.[1,2] The endothelial nitric oxide synthase (eNOS) is enriched in plasma membrane caveolae, where it interacts with caveolin-1 (cav-1), the structural protein of caveolae. This interaction tonically inhibits eNOS enzyme activity and is relieved upon agonist- or stimulus-induced increases in intracellular calcium and the subsequent displacement of cav-1 by the activator calcium–calmodulin complex.[3] Genetic deletion of *cav-1* was shown to result in enhanced endothelium-dependent relaxation of isolated vessels *in vitro*, which was abrogated by NOS inhibition, pointing to a potentiation of NO release from a de-inhibited eNOS.[4] However, this was never formally associated with changes in blood pressure (BP) obtained by conventional measurements, which is at odds with previous reports of increased circulating nitrate/nitrite levels in *cav-1* knockout mice,[5–7] raising doubts about the relevance of the eNOS/cav-1 interaction for BP control. One potential explanation could be partial uncoupling of the de-inhibited eNOS (i.e. producing superoxide anions together with NO), resulting in decreased NO bioactivity *in vivo* (compatible with increased blood nitrate/nitrite levels, which integrate both NO and its oxidation products), as observed in mice with endothelial-specific *NOS3* overexpression.[8] Alternatively, non-conditional deletion of *cav-1* may result in chronic phenotypic adaptation(s) counteracting the effect of enhanced endothelial NO production, or NO regulation of vascular tone may not be a primary regulator of BP homeostasis in these mice *in vivo*. As a few human studies have reported the association of genetic polymorphisms of the *cav-1* gene with systolic hypertension,[9] e.g. in the metabolic syndrome,[10] more studies are warranted to establish the causality of altered cav-1 expression in BP regulation.

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The recent development of miniaturized telemetry implants applicable to the continuous measurement of BP in mice provided new opportunities to approach the dissection of the short-term influence of neurohumoral factors (including NO) on BP. Compared with external sphingomanometry, telemetry provides more sensitive measurements and does not impose stress on the animals, avoiding data artefacts.[11] It is also superior to invasive monitoring of haemodynamics through intra-arterial catheters that require anaesthesia of the animals, a major drawback given the vasoactive and autonomic side effects of most commonly used anaesthetics. In addition to the analysis of BP in the time domain (i.e. profiles over 24 h, with the assessment of the circadian cycle), frequency analysis of complex tracings using the Fourier transformation allows a quantitative measurement of variability at distinctive frequency bandwidths, some of which have been associated with the influence of specific limbs of the autonomic nervous system, at least in rodents.[12] Although a similar frequency analysis of BP or heart rate (HR) variability still needs further validation in man, increases in total variability of BP have clearly been associated with adverse cardiovascular outcomes in patients, emphasizing the clinical relevance of this parameter.[13] Moreover, it has become apparent that, at equal hypotensive efficacy, BP-lowering agents that decrease variability confer additional protection against target-organ damage.[14] Therefore, vasoactive factors may affect variability at unchanged absolute BP levels, with an important bearing on tissue remodelling. This raises the interest for techniques that identify the specific impact of NO on BP variability in animal models of vascular disease, given the well-known protective effects of endothelial NO, e.g. in response to specific drug treatment.

In this study, we use chronically implanted telemetry to identify variability bandwidths in mice that specifically reflect the short-term influence of NO on BP, thereby providing a quantitative measurement of NO-dependent endothelial function *in vivo*. We then apply this method to characterize the phenotype of *cav-1* knockout mice and demonstrate the inverse relationship between variability and circulating NO *in vivo*, or NO production in isolated vessels. Taken together, these data validate the functional importance of cav-1 on the production of bioactive NO and its control of central BP variability and illustrate a quantitative measurement of NO-dependent vascular regulation *in vivo*.

2. Methods

2.1 Experimental protocol

Male mice genetically deficient in caveolin-1 (*cav-1*$^{-/-}$) and their littermate controls (*cav-1*$^{+/+}$) of the same genetic background (C57BL/6; 12–16 weeks old[15,16]) were studied. Animals had free access to water and food, were maintained at 24°C, and kept at a 12 h light/dark cycle.

2.2 Circadian variation and frequency analysis of blood pressure and heart rate by implanted telemetry

BP signals (and HR, derived from pressure waves) from the aortic arch were measured in conscious, unrestrained animals with surgically implanted, miniaturized telemetry devices (Datascience Corp., USA) as described[15,17] (see also Supplement material online). The mortality rates observed after telemetric implantation were <2% and null after the peritoneal catheter implantation. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3 Cell culture

Bovine aortic endothelial cells below passage 10 were cultured in EGM-MV supplemented with 5% serum (Clonetics) in a humidified incubator at 37°C with 5% CO_2, serum-starved for 6 h, and exposed to vehicle (1% DMSO) or a peptide corresponding to the full-length (amino acid 82–101; DGIWKASFTTFTVTKYWFYR; CSD), scaffolding domain of cav-1, synthesized as a fusion peptide to the C-terminus of the Antennapedia internalization sequence (10 μM).[18]

2.4 Protein extraction from aortic samples, co-immunoprecipitation, and western blotting experiments

Mice were anesthetised with a mixture of ketamine/xylasine. Aortas from C57Bl/6, cav-1$^{+/+}$, and cav-1$^{-/-}$ were dissected free from the adventitial tissue and rapidly frozen in liquid nitrogen or incubated in PBS (Cambrex) for 30 min. In some experiments, carbachol (10^{-5} M) was added for the last 2 min. In some experiments, 5 μg/kg of sodium nitroprusside (SNP) was injected to mice 10 min prior to aortic dissection. Immunoprecipitation and western blot analysis were performed as described in the Supplementary material online.

2.5 Nitric oxide assay by electron paramagnetic resonance spectroscopy *in vivo* and *in situ*

The level of circulating Hb-NO as an index of endogenous NO production was determined from analyses of three-line hyperfine components of an electron paramagnetic resonance (EPR) spectra of 5-coordinate-α-Hb-NO complexes after substraction of free radical signals from the EPR spectrum of whole blood as described previously[19] (see also Supplementary material online).

2.6 Vessel reactivity *ex vivo*

Vessel contraction was analysed in aortic segments with a wire myograph, as described in the Supplementary material online.

2.7 Statistical analysis

All results are expressed as mean ± SEM (or SD in the table). All statistical comparisons were performed by use of one-way ANOVA followed by the Dunnett test, or paired and unpaired two-tailed '*t*'-test, where appropriate. To evaluate the influence of drugs on systolic blood pressure (SBP) or very low frequency (VLF) variability and the influence of group on the circadian variation, a two-way ANOVA was performed. We also performed a correlation of Pearson to determine the interaction between VLF of SBP variability and vasodilator-stimulated Ser^{239} phosphoprotein (VASP) phosphorylation. A probability value of $P < 0.05$ was considered significant.

3. Results

3.1 Identification of frequency bands quantitatively reflecting nitric oxide influence on systolic blood pressure variability *in vivo*

We used spectral analysis of SBP tracings from C57Bl/6 mice chronically instrumented with telemetry implants using fast Fourier transformation and isolated the influence of endogenous NO on SBP variability with the combined

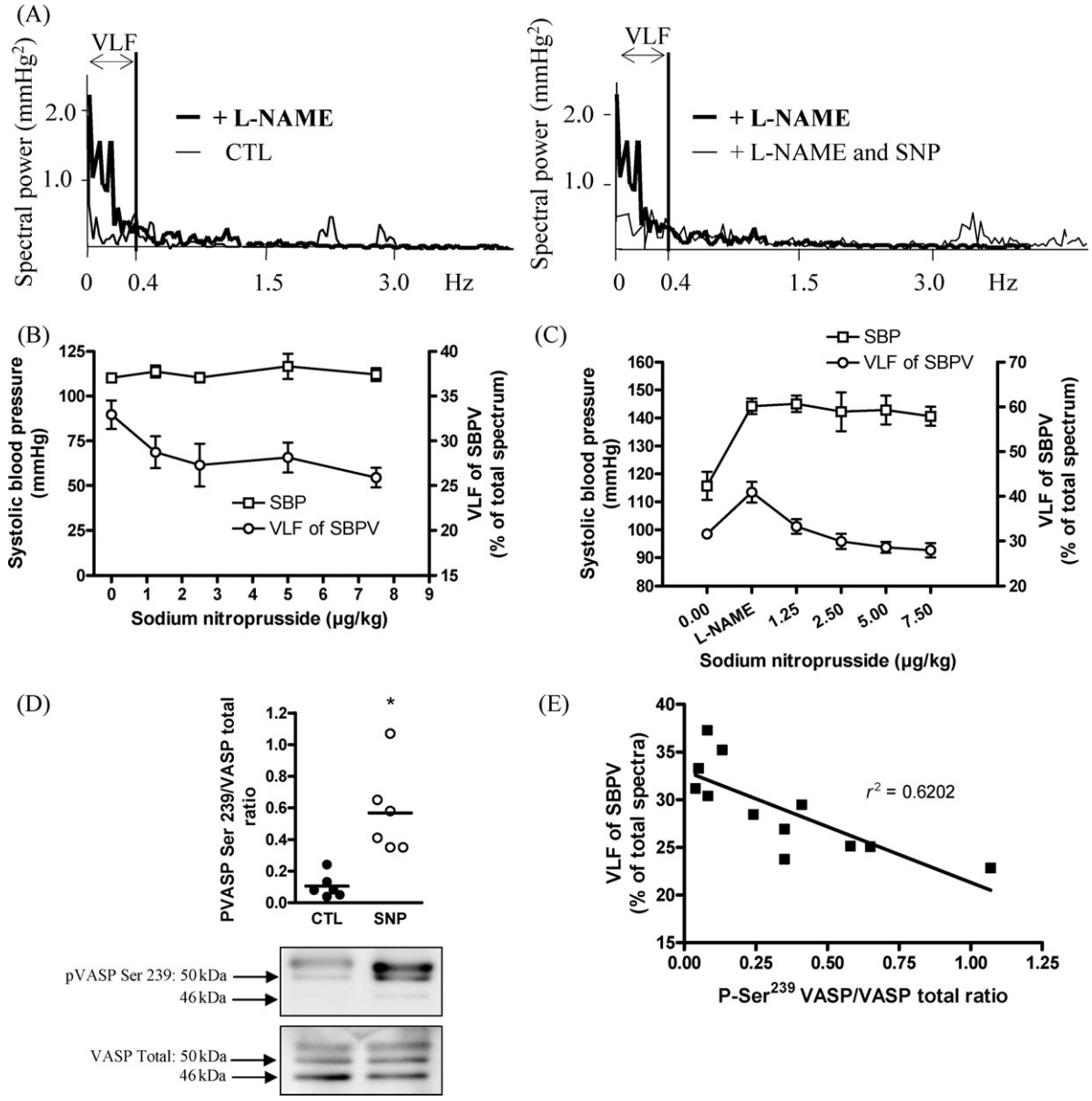

Figure 1 Effect of systemic NO on systolic blood pressure (SBP) variability in the C57Bl/6 mouse; definition of spectral bandwidths and correlation with vascular signalling. (*A*) Power spectra of SBP variability before and after L-NAME in a C57Bl/6 mouse (representative of $n = 5$; $P < 0.01$; left) and after administration of the NO donor, sodium nitroprusside (SNP) in an L-NAME-treated mouse (representative of $n = 5$; $P < 0.05$; right). NO specifically affects spectral bandwidths between 0.05 and 0.4 Hz (defined as very low frequency, VLF). Variability at all other frequencies was unchanged ($n = 5$; $P > 0.05$); Paired '*t*'-test was used for the statistical group comparison. (*B* and *C*) Dose-dependent effect of SNP on SBP and VLF of SBP variability in C57Bl/6 mice ($n = 5$) before (B) and after (C) L-NAME injection (30 mg/kg; ip). VLF variability decreased [$P < 0.05$ and $P < 0.001$ for (B) and (C), respectively] under SNP, at unchanged SBP; For (B) and (C), we performed a two-way ANOVA to evaluate the effect of doses of SNP on the different parameters. (*D*) Representative western blots for Ser^{239}-phosphorylated VASP and total VASP on aortic extracts from C57Bl/6 mice treated with vehicle (control, CTL, $n = 6$) or SNP (5 µg/kg; ip; 10 min; $n = 6$). VASP phosphorylation is reported as $P\text{-}Ser^{239}$ VASP/VASP total ratio. *$P < 0.05$ vs. control by unpaired '*t*'-test. (*E*) Correlation between variability of SBP in the VLF and aortic VASP phosphorylation.

following approaches. First, the effect of NOS inhibition was systematically examined on the entire variability spectra. Acute NOS inhibition by intraperitoneal L-NAME injection resulted in increased variability at frequencies between 0.05 and 0.4 Hz (33.0 ± 0.6 vs. 40.9 ± 2.3, $n = 5$, $P < 0.01$) (*Figure 1A*, left), which we identify as VLFs. Conversely, in L-NAME-treated mice, administration of exogenous NO in the form of sodium nitroprusside (ip; SNP) decreased VLF variability back to levels observed in untreated mice (33.3 ± 1.6, $n = 5$, $P < 0.05$) (*Figure 1A*, right). Importantly, variability <0.05 Hz and at all frequencies >0.4 Hz, up to 5 Hz (normalized to the total spectrum) were not changed, either by L-NAME treatment or SNP (in the presence or absence of L-NAME) ($n = 5$ mice, $P > 0.05$). Since the interpretation of the increased VLF variability upon L-NAME addition may be confounded by the accompanying increase in SBP, dose–response curves to SNP were performed, using a chronically implanted peritoneal catheter connected to a distant syringe (thereby avoiding artifacts due to acute injections and animal manipulations). Small doses of SNP resulted in a proportional decrease in VLF variability ($P < 0.05$),

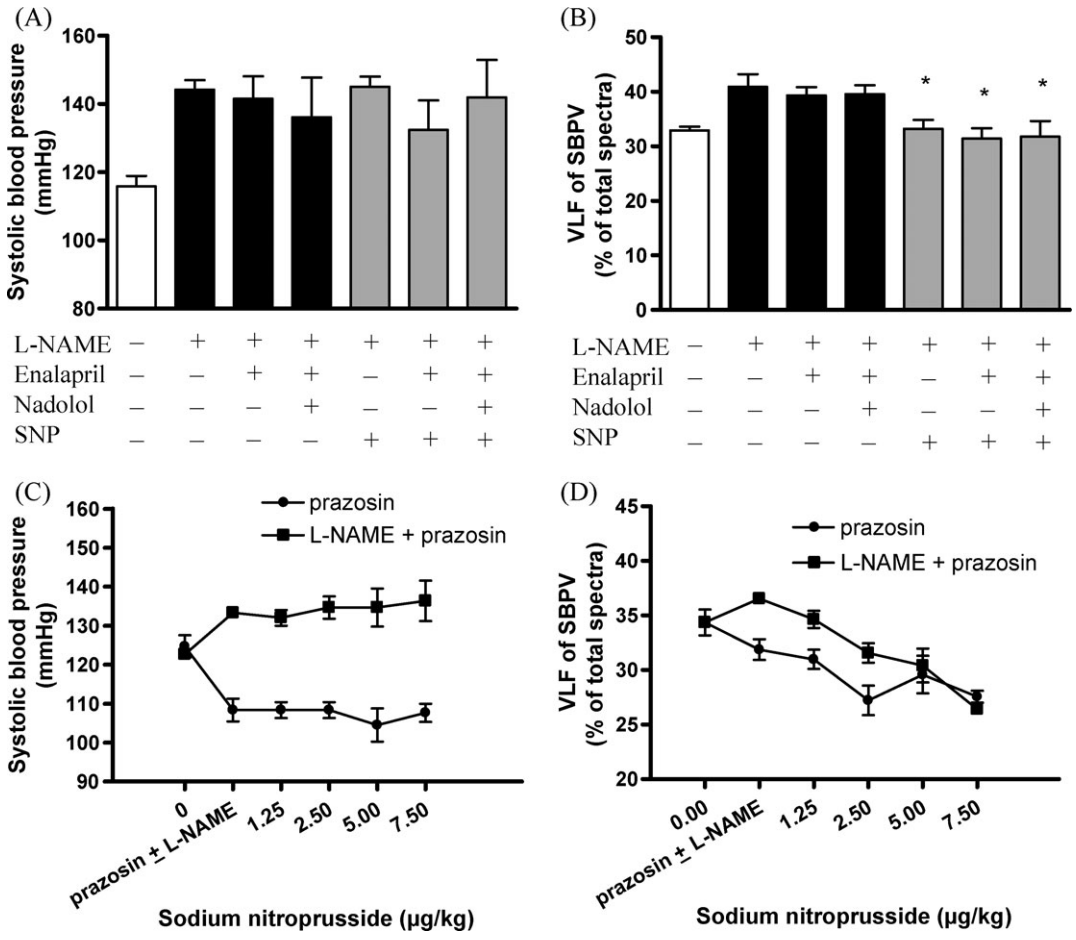

Figure 2 Insensitivity of very low frequency (VLF) variability to adrenergic and renin–angiotensin inhibition at constant blood pressure levels. (*A* and *B*) Systolic blood pressure (A) and VLF variability (B) in WT (C57Bl/6; $n = 5$ for each group) mice injected with L-NAME and sodium nitroprusside (SNP) (1.25 μg/kg) with or without simultaneous pharmacological blockade of the renin–angiotensin system with enalapril or β-adrenergic axis with nadolol. *$P < 0.05$ vs. without SNP; assessed by one-way ANOVA. (*C* and *D*) Effect of increasing doses of SNP on systolic blood pressure (C) and its variability in the VLF (D) in C57Bl/6 mice ($n = 3$) after injection of prazosin or injection of prazosin and L-NAME in combination. After prazosin injection, SNP decreased variability in the VLF [$P < 0.001$; assessed by two-way ANOVA for (D), at unchanged SBP levels (C)].

despite unchanged SBP *in vivo*, and this was also observed after pre-injection of L-NAME prior to SNP ($P < 0.001$) (*Figure 1B* and *C*). VASP phosphorylation has been used as a marker of the activity of the NO/cGMP pathway in vascular tissues.[20] *In vivo* injection of SNP (ip; 5 μg/kg) increased VASP phosphorylation in aortas ($P < 0.05$), and the variability of SBP in the VLF was inversely correlated with VASP phosphorylation, linking this variability with the vascular effects of NO (*Figure 1D* and *E*). To evaluate the contribution of other neurohumoral factors potentially modulated by systemic NOS inhibition or exogenous NO administration, SBP and VLF variability were next examined in mice injected with L-NAME and SNP with or without simultaneous pharmacological blockade of the renin–angiotensin or adrenergic axis. Co-administration by intraperitoneal injection of enalapril (an inhibitor of the angiotensin-converting enzyme) or enalapril with nadolol (a non-specific beta-blocker) neither changed the SBP nor the VLF variability in response to NOS inhibition and its attenuation by SNP (*Figure 2A* and *B*). Furthermore, intraperitoneal injection of prazosin (an inhibitor of α_1 adrenergic receptors) alone leads to a decrease in SBP as well as VLF variability, but did not prevent further decreases in VLF variability with SNP at unchanged SBP ($P < 0.001$) (*Figure 2C* and *D*). A similar prazosin-insensitive reduction of VLF was observed with SNP after L-NAME treatment, at constant SBP (*Figure 2C* and *D*). This excludes a BP-independent contribution of the adrenergic or renin–angiotensin systems in the regulation of SBP variability by systemic NO in the frequency range (0.05–0.4 Hz) identified here as VLF. For all the experiments mentioned above, the collection of data was made after 15 min of injection for L-NAME, enelapril, nadolol, and parzosin and after 5 min of injection for SNP.

3.2 *cav-1*$^{-/-}$ mice have unchanged systolic blood pressure and circadian variation compared with wild-type littermate

Next, BP and HR were compared between *cav-1*$^{-/-}$ and wild-type littermate *cav-1*$^{+/+}$ mice. The SBP and diastolic blood pressure (DBP) profiles of *cav-1*$^{+/+}$ exhibited a physiological circadian variation, with lower pressure during the day (corresponding to the resting period in mice). A similar circadian variation was observed in *cav-1*$^{-/-}$. Their 24 h, as well as night or day mean SBP were also identical to *cav-1*$^{+/+}$, although they had slightly enhanced DBP and HR values ($P < 0.05$) (*Figure 3* and see Supplementary material online, *Table S1*).

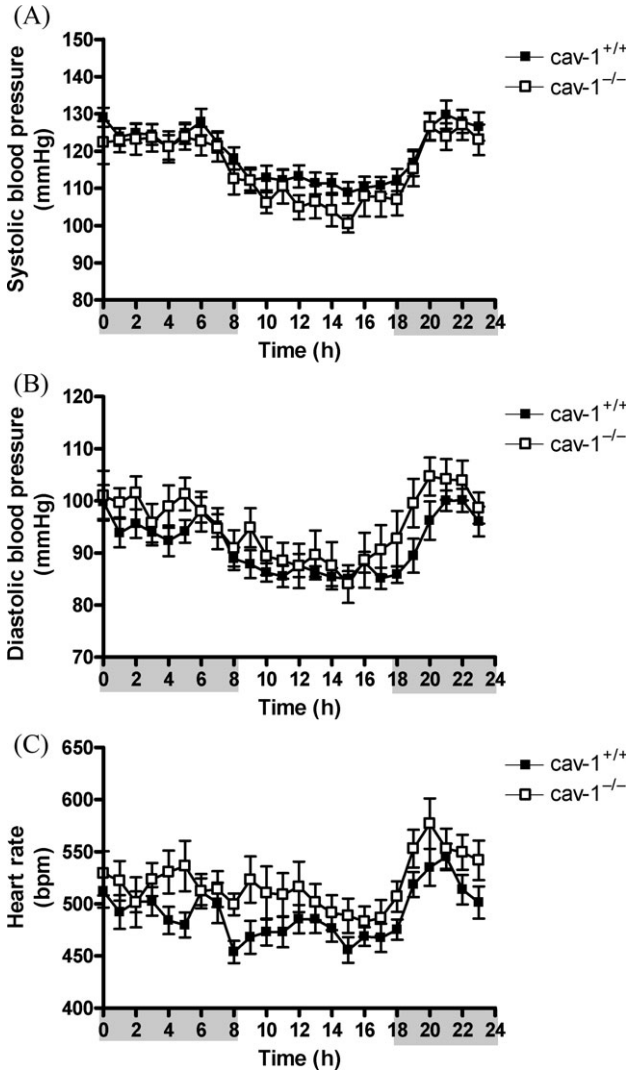

Figure 3 Circadian variation of blood pressure and heart rate in *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice. (*A*) Systolic blood pressure (SBP), (*B*) diastolic blood pressure (DBP) and (*C*) heart rate (HR) were recorded over 24 h in *cav-1*$^{+/+}$ ($n = 15$) and *cav-1*$^{-/-}$ ($n = 13$) mice. Shaded areas on the *X*-axis represent dark cycles (activity period in mice). Mean values ($\pm$SEM) of SBP, DBP, and HR were calculated for each 60 min sequence of recording. To evaluate the influence of group on the circadian variation, a two-way ANOVA was performed, including an interaction term for treatment by time.

3.3 *cav-1*$^{-/-}$ mice have increased circulating nitric oxide levels and calcium-induced nitric oxide production in aorta

To verify that *cav-1* genetic deletion is paralleled with increased NO synthesis and levels of NO in circulating blood *in vivo*, nitrosyl-Hb (Hb-NO) levels were measured in the venous blood by EPR from both mouse strains. Whole blood Hb-NO at baseline was increased by ~88% in *cav-1*$^{-/-}$ when compared with *cav-1*$^{+/+}$ mice (*Figure 4A*). That the Hb-NO content was resulting from active NO synthesis *in vivo* was demonstrated from its decrease in the blood of both *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice that had been treated with L-NAME. Since circulating NO integrates many sources of NO synthesis, we performed a similar comparison in isolated aortas from *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice using EPR spin-trapping of NO *ex vivo*. Calcium-induced production of NO in isolated aortas with the calcium ionophore, ionomycin, induced a 5-fold increase in the NO signal in *cav-1*$^{+/+}$ aortas, which was clearly inhibited by L-NAME (*Figure 4B*). Compared with *cav-1*$^{+/+}$, the NO increase with ionomycin was larger in aortas from *cav-1*$^{-/-}$ mice (up to 7-fold; $P < 0.05$), supporting an enhanced calcium-mediated vascular eNOS activation upon genetic deletion of *cav-1*. We confirmed that such potentiation in *cav-1*$^{-/-}$ mice extended to receptor-mediated activation of NO production and vessel relaxation *in vitro* and *in vivo* (see Supplementary material online, *Results* and *Figure S1*). Importantly, *en-face* staining of the endoluminal side of aortas showed no evident morphological change in the endothelial lining between the two strains (data not shown). Similarly, the abundance of eNOS proteins (by western blotting) was comparable in aortic extracts from *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice (*Figure 4C*).

3.4 *cav-1*$^{-/-}$ mice have a decreased very low frequency variability of systolic blood pressure

The impact of enhanced NO production, at unchanged SBP, was then examined on BP variability in *cav-1*$^{-/-}$ mice. Compared with *cav-1*$^{+/+}$, *cav-1*$^{-/-}$ mice had a significant decrease in VLF variability (*Figure 5A*). To verify if NO was responsible for this difference, mice were next injected (ip injection) with the NO synthase inhibitor, L-NAME. NOS inhibition *in vivo* resulted in increased SBP and VLF variability, as expected (*Figure 5A* and *B*). However, despite a similar absolute increase in SBP in both strains (*Figure 5C*), the increase in VLF variability was more marked in *cav-1*$^{-/-}$ (and L-NAME abrogated the VLF difference between the two groups). Thus, increased vascular NO production in *cav-1*$^{-/-}$ mice translates into less VLF variability *in vivo*.

3.5 Acute inhibition of endothelial nitric oxide synthase with a caveolin scaffolding domain peptide decreased nitric oxide bioavailability and increased very low frequency variability *in vivo* and *in vitro*

To obviate confounding effects of compensatory phenotypic adaptation after non-conditional genetic deletion of *cav-1*, we also examined the effect of an acute intervention that mimics the interaction of cav-1 on eNOS, in the same models as described above. To do this, we used a peptide containing a scaffolding domain of caveolin-1 (amino acids 82–101, CSD) fused to an internalization sequence from Antennapedia, known to reproduce caveolin's inhibitory effect on eNOS, both *in vitro* and *in vivo*.[18] Incubation of endothelial cells with the CSD peptide decreased the amount of eNOS co-immunoprecipitated with cav-1 (*Figure 6A*), consistent with a displacement of the endogenous caveolin–eNOS interaction by the CSD peptide. Dose- and time-dependent effects of intraperitoneal injection of CSD were examined on SBP in *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice. Injection of CSD had no effect on absolute SBP values at 30 min (data not shown). However, frequency analysis of SBP variability showed an increase in the VLF variability as early as 30 min in both *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ (and no effect of the solvent alone) (*Figure 6B*). This effect of a single injection progressively faded away after 24 and 48 h. The effect of CSD was due to an interference with NO synthesis *in vivo*, as ascertained from

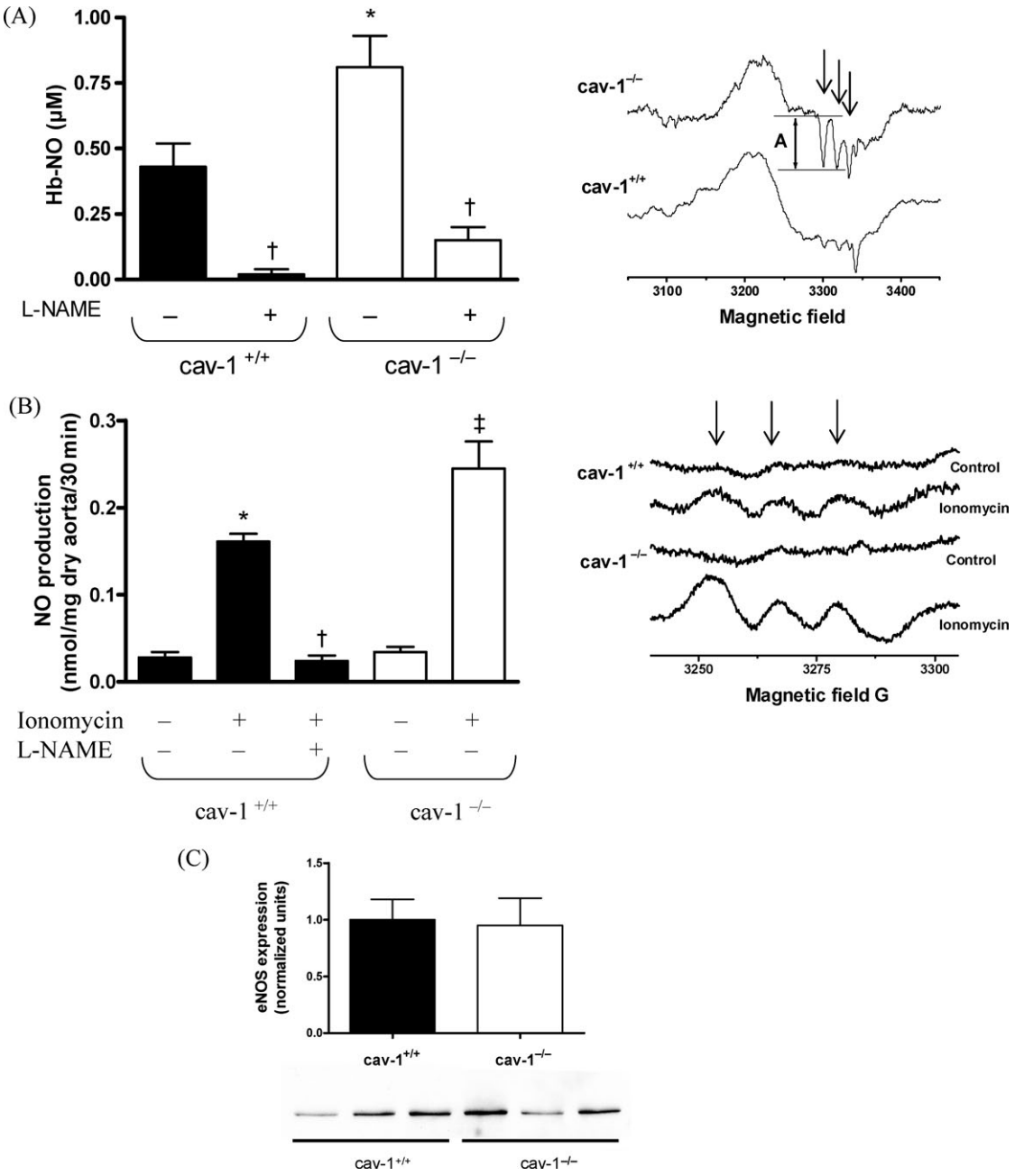

Figure 4 NO production in blood and aorta of *cav1*$^{+/+}$ and *cav-1*$^{-/-}$ mice; effect of NOS inhibition. Blood and aortic tissue were collected from mice previously treated or not with L-NAME. (*A*) Mean concentrations (left) of nitrosyl haemoglobin (5-coordinate-α-Hb-NO) in venous blood measured by electron paramagnetic resonance (EPR) ($n = 4$–9, $^{*}P < 0.05$ vs. cav-1$^{+/+}$ and $^{\dagger}P < 0.05$ vs. no L-NAME). Typical EPR spectra (right) of venous blood from *cav-1*$^{-/-}$ and *cav-1*$^{+/+}$ mice. (A) represents the amplitude; arrows represent hyperfine components of the 5-α-Hb-NO complexes of EPR spectrum. (*B*) Mean concentration (left) of [Fe(II)NO-(DETC)$_2$] complexes in aortas from mice treated or not with L-NAME, and incubated with colloid [Fe(II)-(DETC)$_2$] and ionomycin (2 μM) for 30 min at 37° C. Data are quantified from the EPR signals of [Fe(II)NO-(DETC)$_2$] in aortic rings and normalized to the weight of dry aortas ($n = 3$–4, $^{*}P < 0.05$ and $^{\ddagger}P < 0.01$ vs. without ionomycin, $^{\dagger}P < 0.05$ vs. without L-NAME. Typical [Fe(II)NO-(DETC)$_2$] EPR spectra (right) from isolated aortas from *cav-1*$^{-/-}$ and *cav-1*$^{+/+}$ mice. Arrows indicate the three hyperfine components of the [Fe(II)NO(DETC)$_2$] EPR signal. (*C*) Upper: densitometric data of aortic eNOS expression measured by western blot from *cav-1*$^{+/+}$ ($n = 3$) and *cav-1*$^{-/-}$ ($n = 3$) mice; lower: representative western blot for eNOS in *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice. Unpaired '*t*'-test was used to compare both groups.

additional experiments after L-NAME treatment (ip injection). L-NAME increased SBP variability in the VLF to a similar level in both groups (as expected, *Figure 5A*), but contrary to untreated mice, CSD failed to further increase VLF variability on top of L-NAME (at similar SBP levels), confirming that the effect of CSD depends on a functional NOS (*Figure 6C*). Direct measurements of blood Hb-NO (*Figure 6D*) and NO by EPR spin-trapping in isolated aortas from the CSD-treated mice (*Figure 6E*) confirmed that CSD reduced Hb-NO levels and aortic NO production in both strains. Likewise, ip injection of CSD fully abrogated the BP response to carbachol (96.8 ± 3.4 vs. 67.4 ± 5.6% of baseline SBP in *cav-1*$^{-/-}$ mice with and without CSD peptide, respectively, $n = 5$; $P < 0.05$) and attenuated the increase in phospho-VASP with carbachol in both *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$, consistent with inhibition of eNOS and decreased NO bioactivity after CSD administration (Supplementary material online, *Figure S2*).

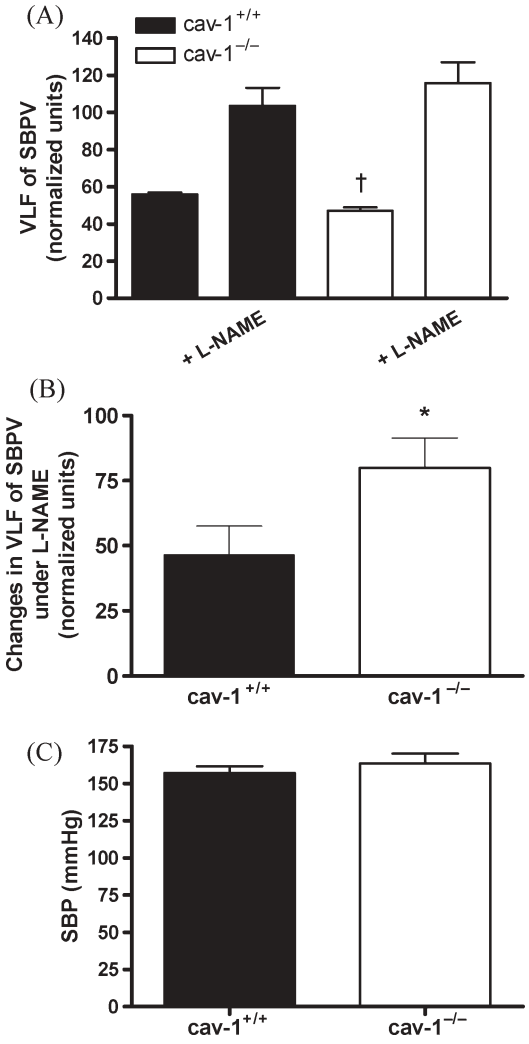

Figure 5 Spectral analysis of systolic blood pressure (SBP) variability in the very low frequency (VLF) domain in *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice. (*A* and *B*) The area under the curve for the variability of SBP in the VLF (0.05–0.4 Hz) was calculated and normalized to whole power spectra for each *cav-1*$^{+/+}$ ($n = 15$) and *cav-1*$^{-/-}$ ($n = 13$) mouse, before (A) and after L-NAME injection (A and B). The effect of L-NAME resulting in similar SBP increase for both groups is also shown (*C*). $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ vs. *cav-1*$^{+/+}$. Unpaired '*t*'-test was used to compare both groups.

4. Discussion

Our data show that *cav-1*-deficient mice have increased circulating Hb-NO *in vivo*, vessel relaxation, and NO production *ex vivo*, as expected from increased activity of eNOS, 'unclamped' from the inhibitory interaction of caveolin-1.[4] Our use of implanted telemetry in awake mice demonstrates that their SBP is unchanged, but spectral analysis identified a decreased variability in specific bandwidths reflecting NO 'buffering' of SBP. Conversely, acute administration of a CSD peptide that reproduces the inhibitory regulation of eNOS by cav-1 produced the opposite effect, i.e. increased SBP variability, both in wild-type and *cav-1*$^{-/-}$ animals. Taken together, our data validate the key role of the allosteric regulation of vascular eNOS by cav-1 *in vivo* and the use of spectral analysis for the quantitative measurement of NO-dependent regulation of BP, applicable for the assessment of various molecular, genetic, or pharmacological interventions, as illustrated with the CSD peptide.

The influence of neurohumoral factors on BP and its variability has been mostly examined in rats and larger animals, but only in limited studies in mice. In the latter, the influence of eNOS was examined by the comparison of BP profiles in *NOS3*$^{-/-}$ mice and their controls (in the same C57Bl/6 background as used by us).[21] Notably, there is good agreement between the frequency bandwidths specifically affected by NOS inhibition in the present study and by *NOS3* deletion. A major limitation of that study was the concurrent elevation of BP in *NOS3*-deficient mice, so that the relative contribution of eNOS activity vs. the chronic hypertension *per se* could not be resolved. Based on the modifications of the power spectra in wild-type (C57Bl/6, WT) mice treated with a NOS inhibitor, we identified frequencies between 0.05 and 0.40 Hz (VLF) to be responsive to acute changes in NO production. We verified that these spectral bandwidths were affected by NO independently from changes in absolute levels of BP by analysing the effect of exogenous NO (low doses of SNP) at unchanged levels of BP. Changes in BP variability did not result from NO-dependent regulation of autonomic nervous system activity or systemic neurohormones, which may affect SBP power spectra in partly overlapping frequencies,[22] since the effect of NO was insensitive to blockade of the renin–angiotensin system or adrenergic systems (or both) at unchanged absolute BP levels. Conversely, the influence of the adrenergic system (e.g. Mayer waves[23]) on VLF is also modulated by NO through post-synaptic effects, as demonstrated from the NOS-dependent attenuation of the vasopressor effect of phenylephrine in *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice (Supplementary material online, *Figure S1D*). However, it is important to note that our definition of NO-regulated VLF in the mouse in this manuscript should not directly be applied to humans. Indeed, the bandwidths of spectral analysis change according to the species and even if some studies suggest a definition for the VLF, LF, and HF variability, more work is needed to identify the effect of NO on these bandwidths in humans.

Spectral analysis of BP variability in *cav-1*$^{-/-}$ mice further validates the influence of NO in the VLF in this species. We first verified that *cav-1* deletion results in endothelial-derived increased vascular NO production through EPR measurements in intact aortic segments of *cav-1*$^{-/-}$ mice *ex vivo*, and, for the first time, in circulating Hb-NO by EPR. In these animals, SBP variability in the VLF was also decreased (reversibly under NOS inhibition), further confirming the 'buffering' effect of NO on SBP variability in our selected bandwidths *in vivo*. From a complete analysis of BP profiles in the time domain, we can exclude independent effects of BP levels or central effects, since SBP was strictly unchanged in *cav-1*$^{-/-}$ when compared with WT, and the circadian regulation was preserved. Our use of a fusopeptide containing a CSD of cav-1, which is sufficient to inhibit eNOS activation, *in vitro* and *in vivo* (*Figure 6*, and ref.,[5] adds several interesting observations; first, its acute administration produced the mirror effect of *cav-1* deletion, i.e. increased VLF variability (at unchanged SBP), and had no effect beyond that of systemic NOS inhibition; this strongly argues in favour of an NOS-dependent regulation of variability by cav-1 and against confounding effects of chronic secondary adaptations in the phenotype of *cav-1*$^{-/-}$ mice. The CSD peptide also abrogated the effects of carbachol on VASP phosphorylation

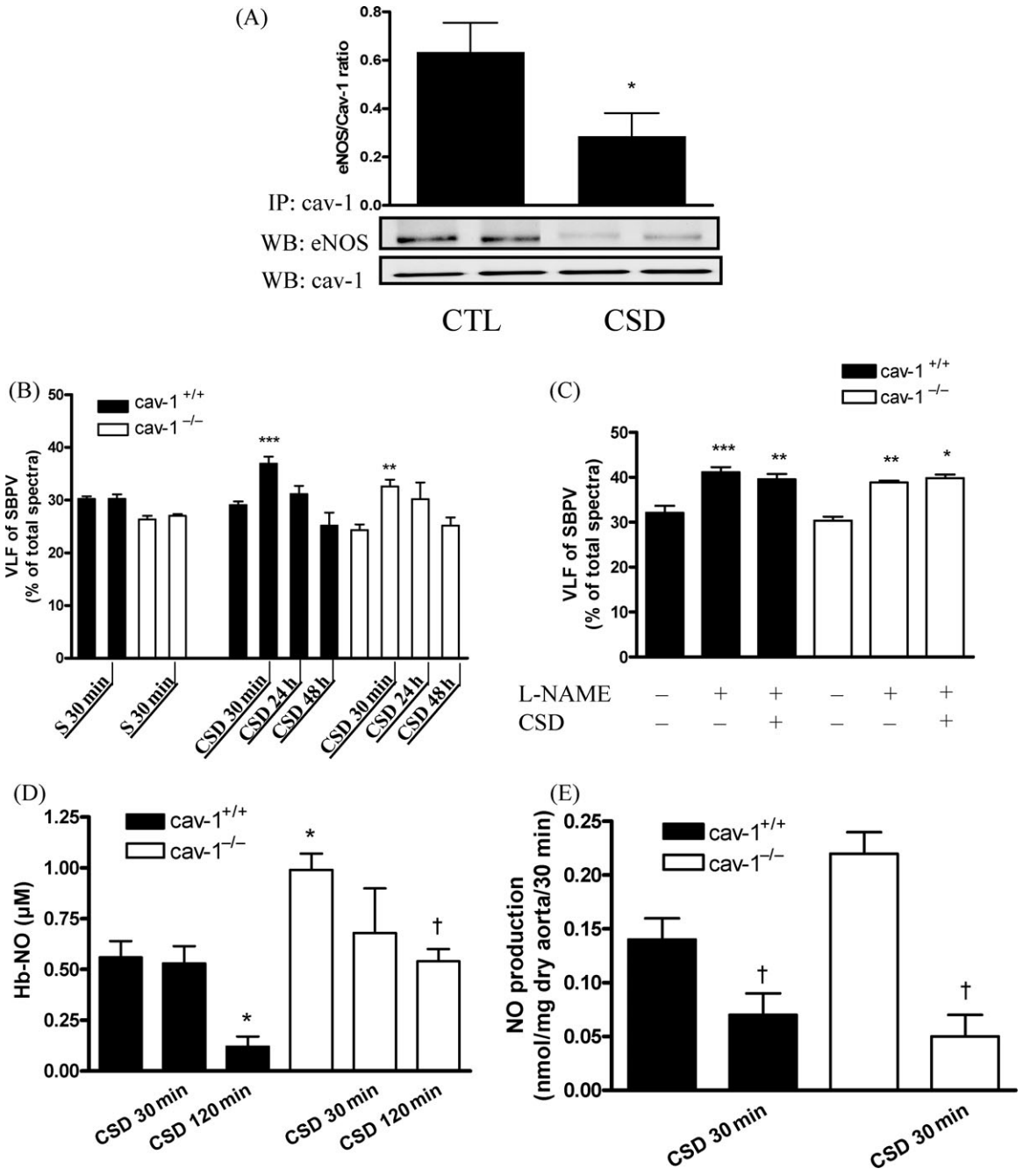

Figure 6 Effect of acute administration of the caveolin scaffolding domain (CSD) peptide *in vitro* and *in vivo*. (*A*) Effect of CSD (10 μM, 6 h) on the interaction of endogenous cav-1 with eNOS in bovine aortic endothelial cells, assessed by co-immunoprecipitation. ip, immunoprecipitation; WB, western blot. Representative western blot and densitometric data from six experiments. $^{*}P < 0.05$ vs. control; assessed by '*t*'-test. (*B* and *C*) Spectral analysis of systolic blood pressure (SBP) variability in the very low frequency (VLF) domain after CSD injection (2.5 mg/kg) or solvent (S; 1% DMSO in physiologic solution). The area under the curve for the variability of SBP was calculated in the VLF (0.05–0.4 Hz) and normalized to whole power spectra for each *cav-1*$^{+/+}$ ($n = 7$) and *cav-1*$^{-/-}$ ($n = 4$) mouse (B) and the effect of NOS inhibition tested after L-NAME injection (C). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. basal level; assessed by one-way ANOVA. (*D*) Levels of nitrosyl haemoglobin (5-coordinate-α-Hb-NO) in venous blood of *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice treated or not with CSD (2.5 mg/kg, ip) for 30 and 120 min. Mean concentrations of Hb-NO measured by EPR and quantified after subtraction of the EPR signal of ceruloplasmin and free radicals ($n = 4$–9, $^{*}P < 0.05$ vs. *cav-1*$^{+/+}$ baseline and $^{\dagger}P < 0.05$ vs. *cav-1*$^{-/-}$ baseline; assessed by one-way ANOVA). (*E*) NO production in isolated aortic rings obtained from *cav-1*$^{+/+}$ and *cav-1*$^{-/-}$ mice treated with CSD (2.5 mg/kg, ip) for 30 min and stimulated with ionomycin *in situ*. Mean concentrations of [Fe(II)NO-$(DETC)_2$] complexes in aortas incubated with colloid [Fe(II)-$(DETC)_2$] and ionomycin (2 μM) for 30 min at 37°C. Data are quantified from the EPR signals of [Fe(II)NO-$(DETC)_2$] in aortic rings and normalized to the weight of dry aortas ($n = 4$–7, $^{\dagger}P < 0.05$ vs. baseline; assessed by unpaired '*t*'-test).

in vivo in both mouse strains. This shows that the CSD peptide can be efficiently delivered to conductance vessels and used as an inhibitor of eNOS signalling *in vivo*. In addition to validating the frequency bands responsive to NO, our use of this CSD peptide also provides key evidence that vascular NO deficiency is a cause of altered BP variability rather than a consequence of high BP producing increased variability *per se*, thereby demonstrating the pathophysiological importance of NO-dependent endothelial function for BP homeostasis.

Our study provides some critical elements extending the understanding of the role of cav-1 on vascular regulation.

Our measurements of increased production of NO by *cav-1*-deficient vessels further validate the functional importance of the negative allosteric modulation of eNOS by cav-1 *in vivo*; they also argue against a potential 'uncoupling' of the enzyme (i.e. shift from NO to superoxide production) upon *cav-1* deletion, in contrast to endothelial *NOS3* transgenic mice,[8] perhaps due to different subcellular localization of eNOS in *cav-1*$^{-/-}$ endothelia.[3,24] In addition to enhancing eNOS activity, *cav-1* deletion may also potentiate NO bioavailability indirectly as a result of attenuating atherogenesis through other mechanisms unrelated to NO.[25] Importantly, the overall increase in detectable NO does not exclude the possibility of stimulus-specific disruption of compartmentalized eNOS signalling. *cav-1*-deficient animals lack caveolae in their endothelium, which we have shown to be associated with defective coupling of vascular endothelial growth factor-receptor 2 (VEGF-R2; or Flk-1) to eNOS and impaired NO-dependent angiogenesis.[24] Recently, gross alterations in chronic shear–stress-induced vascular remodelling and NO signalling were reported in *cav-1*-deficient mice, further emphasizing the importance of endothelial caveolae for mechanosensing, and perhaps, eNOS internalization for signalling to intracellular compartments.[26] Loss of these specialized modes of signalling may account for the surprising absence of hypotension in *cav-1*$^{-/-}$ mice, despite enhanced vascular NO production in response to stimuli that do not need caveolae for signalling, such as acetylcholine and α_1-adrenergic agonists. One could argue that most of our *ex vivo* work used large conductance vessels which may be less critical for the regulation of BP *in vivo* than resistance arteries. Nevertheless, we found NO-dependent relaxation to be equally potentiated in mesenteric arteries from *cav-1*$^{-/-}$ mice. Still, BP is unchanged in *cav-1*$^{-/-}$ mice, which could be explained by their complete loss of EDHF signalling, known to be functionally prevailing in resistance arteries. We found this to be secondary to a mislocalization of TRPV4 and connexins, both critical for the EDHF response.[27] Therefore, although the reciprocal influence of cav-1 on NO and EDHF in resistance vessels may explain the resultant neutral effect on absolute BP levels in *cav-1*$^{-/-}$ mice, the enhanced NO production may translate into less BP variability through a predominant effect in conductance vessels, where the NO pathway prevails.

In conclusion, our study highlights the role of cav-1 in the regulation of BP *in vivo*, an important determinant of cardiovascular morbidity and prognosis,[13,28] and raises the interest for genetic, molecular, or pharmacological interventions targeting cav-1 for the treatment of NO-dependent endothelial function in diseases such as hypertension and atherosclerosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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