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Impact of bradykinin type 2 receptor overexpression on cardiovascular function in mice

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ABSTRACT

Bradykinin is an endogenous peptide playing an important role in regulation of vascular function and permeability, but cardiovascular effects of endogenous bradykinin remain incompletely understood. We studied whether genetically engineered overexpression of endothelial bradykinin type 2 receptor (B_2) alters cardiac and vascular function using mice with endothelial-specific overexpression of B_2 (B_2^{tg}) and their transgene-negative littermates (B_2^n). In the aorta, brain-stem and skeletal muscle of B_2^{tg} human BDKRB2 were exclusively expressed, and B_2-overexpression was evident by a significant upregulation of B_2 protein. Endothelium-dependent aortic relaxation and responses to phenylephrine or the nitric oxide (NO) donor measured *ex vivo* were unaffected by the transgene. In contrast, bradykinin induced constriction in B_2^n, but i vasorelaxation in B_2^{tg} that was sensitive to inhibition of NO-synthase (NOS). *In vivo* assessment of endothelial function by flow-mediated dilation revealed no changes in B_2^{tg} as compared to B_2^n. Likewise, transthoracic echocardiography showed no effect of B_2 overexpression on cardiac function. In contrast, decreased systolic blood pressure and bradycardia were observed in B_2^{tg}. The decrease in blood pressure in B_2^{tg} remained following inhibition of cyclooxygenase and NOS, but was completely abolished by the selective B_2 antagonist icatibant. Thus, endothelial-specific B_2-overexpression induced mild hypotension and bradycardia while having no effect on flow-mediated dilation or several cardiac function parameters. These minor effects of endothelial-specific B_2 overexpression in healthy mice suggest that alterations of endogenous bradykinin generation may not be associated with derangements of cardiovascular functions, however, the long-term safety of continuous changes of bradykinin in cardiovascular comorbidities deserves further rigorous clinical investigation.

1. Introduction

The nonapeptide bradykinin is produced in the circulation of various mammalian species, including humans, predominantly by proteolysis of high molecular weight kininogen by kallikrein [1], and is rapidly degraded by the zinc metalloprotease angiotensin-converting enzyme (ACE) [2]. Bradykinin is well known to induce acute cardiovascular effects, particularly vasodilation and an increase in endothelial permeability. These acute effects of bradykinin are mediated almost exclusively via bradykinin type 2 receptors (B_2), which, in contrast to bradykinin type 1 receptors (B_1), are constitutively expressed in vascular cells such as smooth muscle and endothelial cells [3]. The specific B_2

Abbreviations: ACE, Angiotensin-converting enzyme; ANOVA, Analysis of Variance; B_1, Bradykinin type 1 receptors; B_2, Bradykinin type 2 receptors; B_2^{tg}, Transgenic B_2 mouse line with endothelial-specific overexpression of human B_2; B_2^n, Negative littermates of B_2^{tg}; *BDKRB2*, Bradykinin type 2 receptors human gene; *Bdkrb2*, Bradykinin type 2 receptors murine gene; Bpm, Beats per minute; BW, Body weight; CO, Cardiac output; DEA/NONOate, Diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate; E/A, Early diastolic filling wave/atrial contraction wave; ECG, Electrocardiogram; EF, Ejection fraction; eNOS, Endothelial nitric oxide synthase; FMD, Flow-mediated dilation; FS, Fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *Hprt1*, Hypoxanthine phosphoribosyltransferase 1 murine gene; i.p., Intraperitoneal injection; IVCT, Isovolumic contraction time; IVRT, Isovolumic relaxation time; kD, Kilodaltons; L-NAME, N^{ω}-Nitro-L-arginine methyl ester; LV, Left ventricular; LVPW;d, Left ventricular posterior wall thickness during diastole; LVPW;s, Left ventricular posterior wall thickness during systole; MV DT, Mitral valve deceleration time; ns, not significant; NO, Nitric oxide; NOS, Nitric oxide synthase; PBS, Phosphate buffered saline; PW, Pulsed Wave; SEM, Standard Error of Mean; SV, Stroke volume; WT, Wild-type mice.

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antagonist Icatibant can competitively block such acute effects of exogenously supplied bradykinin in mice [4] and humans [5]. It is useful for the treatment of hereditary angioedema (Online Mendelian Inheritance in Man #106100) and approved for this indication [6]. This rare and potentially lethal form of non-allergic angioedema is mainly triggered by a local endogenous overproduction of bradykinin [7]. On the other hand, ACE inhibitors also induce non-allergic angioedema [8], which in this case is based on the disruption of bradykinin degradation and can also be treated with icatibant [9]. Even though both vasodilation and an increase in endothelial permeability are significantly involved in the development of non-allergic angioedema, icatibant, in contrast to infusion with bradykinin, has no effect on blood pressure in humans [6]. In addition, several studies on B_2-deficient mice also showed no change or a small increase in blood pressure at rest, but a clear inhibition of blood pressure reduction after infusion of bradykinin [10–14].

In recent clinical studies evaluating new drugs for the prophylaxis of acute attacks in hereditary angioedema long-term continuous inhibition of kallikrein and, hence, the generation of endogenous bradykinin was desired. This applies to the approved drugs lanadelumab and berotralstat. In addition, gene therapy approaches have been developed [15–17]. However, endogenous bradykinin is known to exert cardiovascular protective activity [18]. Likewise, it can reduce the infarct area [19] and has a growth inhibitory effect on cardiomyocytes [20]. These activities likely contribute to the beneficial effect of ACE inhibitors for cardiovascular patients [21,22]. Hence, the effects of endogenous bradykinin on blood pressure and other cardiovascular functions deserve further exploration. Therefore, we aimed to investigate cardiovascular effects of endogenous bradykinin in wild-type mice compared to mice harbouring an endothelial-specific B_2 overexpression [4]. Our main findings are that transgenic mice showed minor reductions of heart rate and blood pressure with no other vascular and cardiac alterations.

2. Methods

2.1. Mice

The transgenic B_2 mice with endothelial-specific overexpression of human B_2 (B_2^{tg}) and controls (transgenic negative littermates, B_2^{n}) or wild-type (WT) C57BL/6 (JANVIER LABS, Le Genest-Saint-Isle, France) were used in this study. To generate a transgenic line with endothelial-specific overexpression of B_2, the cDNA of the BDKRB2 was first obtained. For this purpose, the established squamous cell carcinoma cell line 2 (squamous carcinoma cells, Department of Otorhinolaryngology, Heinrich Heine University of Düsseldorf) was used, which expressesB_2. After isolating the RNA and transcribing it into cDNA, it served as a template for the designed gene construct. The human BDKRB2 cDNA was cloned into a pBluescript II SK + vector (Stratagene, Amsterdam, Netherlands), in which the Tie-2 promoter (2.1 Kb), the Lac Z gene (3.1 Kb), the SV40 poly-A signal and the enhancer construct (10 Kb) had already been cloned. The plasmid was originally provided as a LacZ construct by Thorsten Schläger, Max Planck Institute, Bad Nauheim [23] and previously used for the generation other transgenic mice [24,25] in which endothelial-specific overexpression of the target protein was proven by confocal microscopy [26,27]. In cooperation with Dr. Oliver Lieven and Prof. Dr. Ulrich Rüther, Institute for Animal Developmental and Molecular Biology, Heinrich Heine University, the construct was microinjected in fertilized F2-eggs of F1 (C57BL/6 × C3H/He) mice. Microinjected eggs were transferred into the oviducts of pseudo-pregnant mice and allowed to develop to term [4]. The transgenic B_2^{tg} line including B_2^{n} was established and backcrossed to C57BL/6 for more than 20 times. Mice were bread in the animal facility of the University Hospital Düsseldorf, housed in groups (2–5 mice per cage) and kept in specific pathogen free environment with 12-hour light/dark cycle. They had free access to food and water which was acidified to minimize infections (pH = 3). Room temperature and relative humidity were kept at 24–26 °C and 55 ± 5 % respectively. Males of 3–4 months old (25–30 g) were used for experiments. To avoid the possible influence of the oestrous cycle on responses of transgenic female mice to the experimental conditions, we chose to investigate male mice only. The study was conducted according to the German Animal Welfare Act "Tierschutzgesetz" (approval ID: 81–02.04.2018.A354) and the 'Guide for the Care and Use of Laboratory Animals' of the US National Institutes of Health. For organ harvesting, mice were euthanized by carbon dioxide inhalation.

2.2. Quantitative real-time PCR

Aortic, brain stem and skeletal muscle (*M. gastrocnemius*) tissues were dissected and snap frozen in liquid nitrogen. Tissues were stored at −80 °C. Total RNA was isolated using RNeasy® Protect Mini Kit (Qiagen, Hilden, Germany). cDNA was reversely transcribed from 500 µg RNA by using QuantiTect® Reverse Transcription kit (Qiagen, Hilden, Germany). mRNA expression of human and murine bradykinin type 2 receptors (*BDKRB2* and *Bdkrb2)* were identified using Taqman® gene expression assay (Applied Biosystems, Weiterstadt, Germany). Assay IDs for human *BDKRB2* and murine *Bdkrb2* were Hs00176121, Mm01339907 respectively. For normalization, murine hypoxanthine phosphoribosyltransferase 1 (*Hprt1*, Mm03024075) was simultaneously identified (Applied Biosystems, Weiterstadt, Germany). Quantitative real-time PCR experiments were conducted using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Weiterstadt, Germany). *BDKRB2* and *Bdkrb2* mRNA expression relative to *Hprt1* were determined using the ΔCt method.

2.3. Western blotting

Protein lysis was prepared by pulverizing and homogenizing the tissues in cold radioimmunoprecipitation assay buffer mixed with a protease inhibitor cocktail set III (Merck, Darmstadt, Germany), followed by brief sonication and finally centrifugation at 4 °C. Protein concentration was determined by Bradford Protein Assay Kit (Sigma Aldrich, Munich, Germany). Laemmli buffer and a reducing agent β-mercaptoethanol (Thermo Fischer Scientific, Meerbusch, Germany), were added to the sample lysate, and then proteins (30 µg for left ventricular (LV) tissues or 60 µg for aortic tissues) were loaded onto 10 % sodium dodecyl sulfate polyacrylamide gels (hand-casted using acrylamide/bisacrylamide (37.5:1) (Sigma Aldrich, Munich, Germany), 10 µg/ml ammonium persulfate (Sigma Aldrich, Munich, Germany) and 1 µl/ml tetramethylethylenediamine (Sigma Aldrich, Munich, Germany) and transferred onto nitrocellulose membranes (pore size 0.45 µm Millipore, Schwalbach, Germany). After blocking the membranes using 1 % casein (Carl Roth, Karlsruhe, Germany) in phosphate-buffered saline (PBS), incubation was done with the anti-BDKRB2 rabbit monoclonal antibody (1:1000, Invitrogen, Rockford, USA) and a rabbit monoclonal antibody detecting glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10,000, Cell Signaling Technology, Danvers, Massachusetts, USA) for loading control. Finally, protein detection was achieved by labelling with IRDye 800CW goat anti-rabbit IgG (LI-COR Biosciences GmbH, Bad Homburg, Germany) and imaging on Odyssey Infrared Imager 9120 (LI-COR Biosciences Nebraska, USA). Captured Images were quantified by Image Studio™ Lite software Ver 5.2 (LI-COR Biosciences, Nebraska, USA).

2.4. Aortic reactivity studies

Freshly isolated thoracic aortas were dissected from mice. Wire-mounted aortic rings (3–4 mm) were placed into the organ bath filled with 10 ml of freshly prepared Krebs-Henseleit buffer (pH = 7.4), supplied with 95 % O_2/5% CO_2 and kept at 37 °C. Aortic rings were exposed to 80 mM potassium chloride (Carl Roth, Karlsruhe, Germany) to check the vascular smooth muscle integrity and to normalize the aortic rings

for different size and muscular content. Intact endothelium was tested by maximal relaxation to acetylcholine (Sigma Aldrich, Munich, Germany); rings with less than 80 % relaxation to acetylcholine were excluded from analysis. After washing and pre-constriction with phenylephrine (Merck, Darmstadt, Germany, 0.2 μM), cumulative concentration–response curves were obtained for acetylcholine (1 nM – 100 μM) and bradykinin (Merck, Darmstadt, Germany, 1 nM – 10 μM). In some experiments, preincubation of aortic rings with non-selective cyclooxygenase inhibitor diclofenac (Sigma Aldrich, Munich, Germany, 10 μM) or with non-selective NOS inhibitor N^{ω}-Nitro-L-arginine methyl ester (L-NAME, Sigma Aldrich, Munich, Germany, 100 μM) was done for 20 min before pre-constriction with phenylephrine (0.2 μM). The vasoconstrictor response to phenylephrine was evaluated by constructing a concentration response curve using increasing concentrations from 1 nM to 10 μM. After a stable plateau of constriction at the latter concentration was reached a concentration response curve for the NO donor diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA NONOate, Enzo, Lörrach, Germany) was performed.

2.5. Systolic blood pressure and heart rate measurement

Systolic blood pressure and heart rate were measured in awake 3–4 months old mice using an automated tail cuff system (Visitech Systems, Apex, North Carolina) as described previously [28]. Mice underwent adaptation period of 7 days preceded counted measurements. In each mouse recordings were made following 3 consecutive rounds of 10 separate measurements each day and measurements for a total of 7 days were summarized to obtain the systolic blood pressure and heart rate values for each mouse. Diclofenac (Sigma Aldrich, Munich, Germany, 10 mg/kg body weight (BW)/day) was administered in drinking water for 3 days and water was changed daily. After assessment of the effects of diclofenac on blood pressure and heart rate, a washout period of one week was included to ensure that the effect of diclofenac had completely disappeared. Afterwards an adjustment period of 4 days of intraperitoneal (i.p.) PBS injection was performed to exclude any effects of i.p. injection *per se* on systolic blood pressure and heart rate. During this adaptation phase, i.p. PBS was injected twice with an interval of 30 min. Then, a specific B_2 antagonist icatibant (Shire, Dublin, Ireland, 1 mg/kg BW/day) was administered i.p. for two days. Measurements were conducted after 30 min of icatibant administration. In some experiments mice were orally treated with L-NAME (L-NAME, Sigma Aldrich, Munich, Germany) for 7 days (100 mg/kg/BW). The drinking water was replaced daily. Selected doses, route and frequency of the drugs administration was based on previously published data [4,29].

2.6. Flow-mediated dilation

Imaging of the external iliac artery was performed using Vevo 3100 with a 29–71 MHz transducer MX700 (VisualSonics Inc, Toronto, Ontario, Canada). Mice were kept under slight mask anaesthesia by an inhaled mixture of 1.5–2.0 % isoflurane and 95 % O_2/5% CO_2. The artery was identified by its characteristic blood flow pattern using colour and pulsed wave (PW) Doppler. A vascular occluder (8 mm diameter, Holly Specialty Product, California, USA) was placed around the lower limb. For measurements of flow-mediated dilation (FMD), first baseline B-mode images and PW Doppler signal of the external iliac artery were recorded, afterwards the cuff was inflated to 200 mmHg, and pressure was kept constant for 5 min to occlude the artery. Then, the occluder was deflated to induce an increased blood flow and FMD. The images were captured every 20 s for 2 min and every 30 s for another 3 min after cuff deflation, i.e. during the reperfusion phase. Vessel diameter was measured for every time point using Brachial Analyzer software version 6 (Medical Imaging Applications LLC, Coralville, USA). Changes in vessel diameter were calculated as percent ratio (%) = [(diameter at each time point − diameter baseline) / diameter baseline] x 100 by two operators blinded to the experimental groups. Mean flow velocity in the artery was calculated from average of three values at each time point using Vevo Lab software version 5.7.1 (Visual Sonics Inc., Toronto, Ontario, Canada).

2.7. Transthoracic echocardiography

Transthoracic echocardiography was done under slight mask anaesthesia by an inhaled mixture of 1.5–2.0 % isoflurane and 95 % O_2/5% CO_2. Electrocardiogram (ECG) was obtained via built-in ECG electrode contact pads. The body temperature was maintained at 36.5–37.5 °C. Heart rate was kept in a range of 450–550 beats per minute (bpm) and respiration rate at 80–120 breaths per minute. Cardiac imaging was performed by using a high-resolution ultrasound transducer MX400 (20–46 MHz; Vevo 3100, Visual Sonics Inc., Toronto, Ontario, Canada), and the manufacturer's analysis software. Parasternal long- and short-axis views and a four-chamber view were acquired. LV volume at end of systole (volume;s), LV volume at end diastole (volume;d), stroke volume (SV), cardiac output (CO), fractional shortening (FS) and ejection fraction (EF) were calculated from B-mode images by identification of maximal and minimal cross-sectional area by an operator blinded to experimental group assignment. LV mass, LV posterior wall thickness during systole (LVPW;s) and diastole (LVPW;d) were calculated from parasternal long axis view of the heart acquired in M−mode. LV mass, LVPW;s and LVPW;d were further normalized forBW. LV diastolic function was measured by analysing the characteristic flow profile of the mitral valve Doppler, which was visualized in apical four-chamber view. Analysis of images was done using Vevo Lab software version 5.7.1 (Visual Sonics Inc., Toronto, Ontario, Canada). Parameters were measured from three different cardiac cycles and average values were calculated

2.8. Statistical analysis

Data analysis was done by GraphPad Prism software version 8.0.2 (San Diego, CA, USA). Data were summarized as mean ± standard error of mean (SEM), and number of biological replicates (n). D'Agostino & Pearson test, Shapiro-Wilk test or Kolmogorov-Smirnov test were used to test the normal distribution of data. Rout test was used to identify outliers. Comparison between two groups was done by unpaired *t*-test. Comparison within groups was done by paired *t*-test. Comparison between more than two groups was done by one-way ANOVA followed by post-hoc Sidak's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparisons test (in case data were not normally distributed) if P-value was significant. Two-way repeated measures ANOVA was used for comparisons between groups across different concentrations or time followed by post-hoc Tukey's or Dunnett's multiple comparisons test if P-value was significant. P-value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of human BDKRB2 mRNA in B_2^{tg} mice

Since human *BDKRB2* gene was introduced in the B_2^{tg} mice, quantitative real time PCR was done to both confirm the expression of the inserted human *BDKRB2* gene and to address whether the insertion of the human *BDKRB2* gene affect the expression of the murine *Bdkrb2* mRNA. Aortic and skeletal muscle tissues were used as a representation of conductive and resistance blood vessels, respectively, brain stem was chosen to assess endothelial B_2 overexpression in central nervous system. As expected, mRNA of human *BDKRB2* was exclusively found in B_2^{tg} mice and was not detected in B_2^{n} mice (Fig. 1). Also, the insertion of the human *BDKRB2* gene did not affect the mRNA expression levels of the constitutive murine *Bdkrb2*. Of note, mRNA expression levels of human *BDKRB2* were significantly higher than that of murine *Bdkrb2* in aortic, skeletal muscle and brain stem tissues of B_2^{tg} mice. Collectively, these

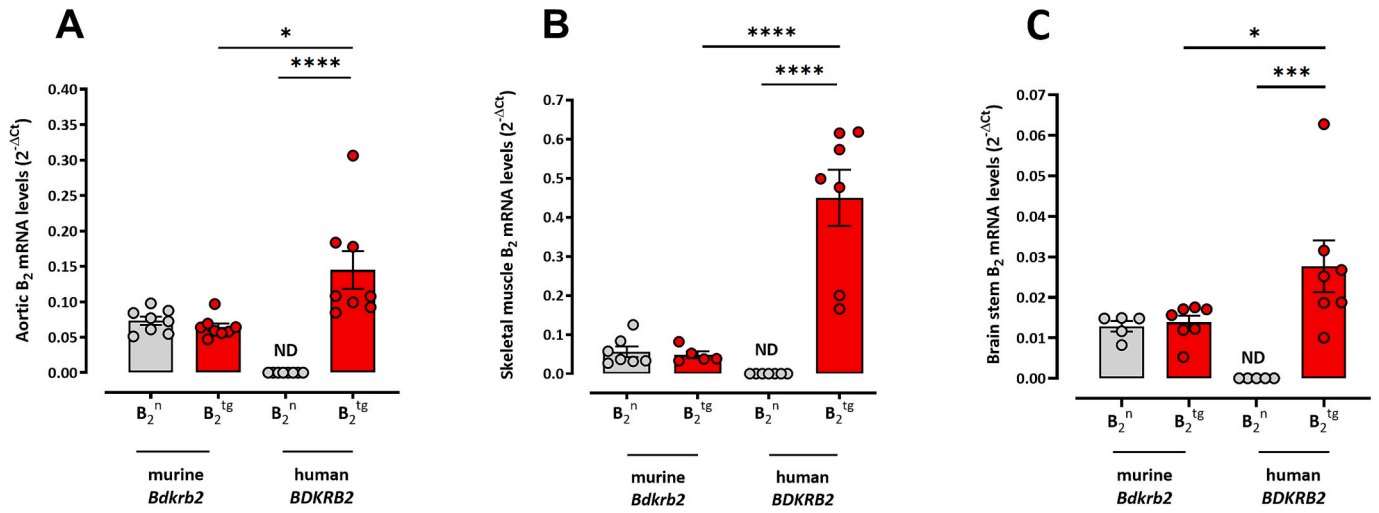

Fig. 1. Quantitative rt-PCR data showing human *BDKRB2* and murine *Bdkrb2* mRNA expression in various tissues of B_2^{tg} and B_2^{n} mice. (**A**) In aortic tissues, human *BDKRB2* mRNA were found only in B_2^{tg} mice (0.145 ± 0.027) and not detectable in B_2^{n} mice (n = 8 per group, ****P < 0.0001). There was no significant difference in constitutive murine *Bdkrb2* mRNA expression levels between B_2^{tg} and B_2^{n} mice (B_2^{n}: 0.073 ± 0.005, B_2^{tg}: 0.064 ± 0.005, n = 8 per group, ns). In B_2^{tg} mice, human *BDKRB2* (0.145 ± 0.027) expression levels were significantly higher than the murine *Bdkrb2* (0.064 ± 0.005) expression levels (n = 8 per group, *P = 0.02). Statistical analysis was done by Dunn's multiple comparisons test following Kruskal-Wallis test. (**B**) In skeletal muscles tissues, human *BDKRB2* mRNA were found only in B_2^{tg} mice (0.45 ± 0.07) and not detectable in B_2^{n} mice (n = 7 per group, ****P < 0.0001). There was no significant difference in the constitutive murine *Bdkrb2* mRNA expression between B_2^{tg} and B_2^{n} mice (B_2^{n}: 0.056 ± 0.014, B_2^{tg}: 0.049 ± 0.008, n = 5–7 per group, ns). In B_2^{tg} mice, human *BDKRB2* expression levels were significantly higher than the murine *Bdkrb2* expression levels (human: 0.45 ± 0.07, murine 0.049 ± 0.008, n = 5–7 per group, ****P < 0.0001). Statistical analysis was done by Sidak's multiple comparisons test following one-way ANOVA. (**C**) In brain stem tissues, human *BDKRB2* mRNA were found only in B_2^{tg} mice (0.028 ± 0.006) and not detectable in B_2^{n} mice (n = 5–7 per group, ***P = 0.0003). There was no significant difference in the constitutive murine *Bdkrb2* mRNA expression between B_2^{tg} and B_2^{n} mice (B_2^{n}: 0.013 ± 0.001, B_2^{tg}: 0.014 ± 0.002, n = 5–7 per group, ns). In B_2^{tg} mice, human *BDKRB2* expression levels were significantly higher than the murine *Bdkrb2* expression levels (human: 0.028 ± 0.006, murine 0.014 ± 0.002, n = 7 per group, *P = 0.04). Statistical analysis was done by Sidak's multiple comparisons test following one-way ANOVA. ND: not detected. *Bdkrb2*: bradykinin type 2 receptors murine gene, *BDKRB2*: bradykinin type 2 receptors human gene.

data confirm the B_2 overexpression in B_2^{tg} mice only (Fig. 1).

3.2. Vascular and cardiac expression of B_2 protein

In a further approach we wished to comparably evaluate B_2 protein expression. To accomplish this, we used a rabbit antibody showing mainly two signals between 70 and 100 kilodaltons (kD) which is in line with two splicing variants described previously [30]. In western blots of murine brain endothelial cells two signals of the same size appeared as well, while in western blots of human dermal vascular endothelial cells

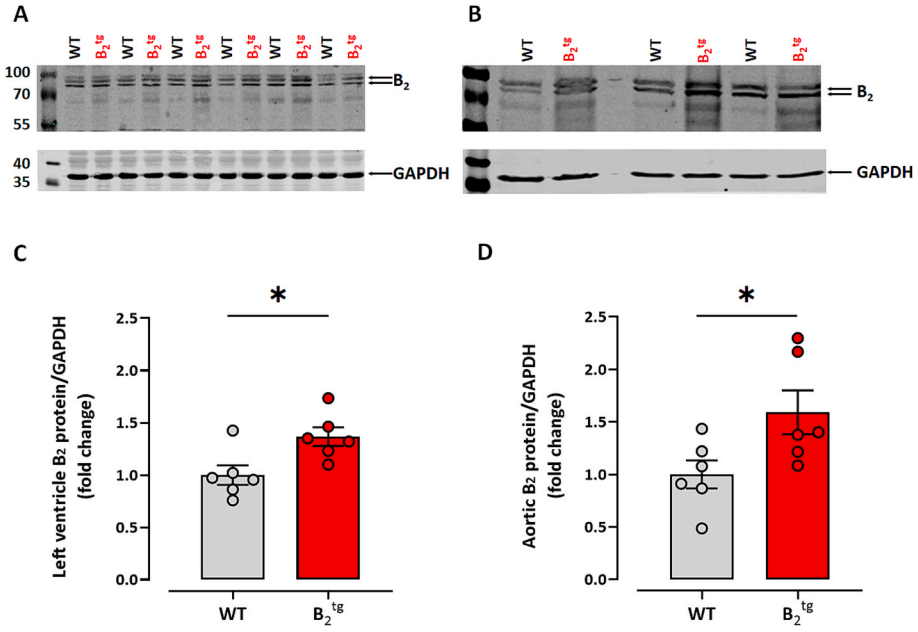

Fig. 2. Evaluation of B_2 protein expression by western blot, including both murine and human B_2 protein in **(A)** left ventricular (30 μg/lane) and **(B)** aortic tissue (60 μg/lane) of B_2^{tg} and wild-type mice (WT). Both signals of B_2 detected between 70 and 100 kDa were calculated in relation to GAPDH signals. B_2 protein expression levels were significantly increased as compared to the expression level detected in wild-type mice **(C)** in left ventricular tissues of B_2^{tg} mice (1.36 ± 0.08 fold, n = 6 per group, *P = 0.01) and **(D)** in aortic tissues of B_2^{tg} mice (1.59 ± 0.20 fold, n = 6 per group, *P = 0.03). Statistical significance was evaluated by unpaired two-tailed *t*-test.

only the lower signal of about 70 kD in size was detected (data not shown). The results shown in Fig. 2 roughly resemble the quantitative real time PCR data and demonstrate an increased B_2 protein expression in left ventricular and aortic tissue of B_2^{tg} compared to WT mice. However, the antibody apparently doesn't discriminate between murine and human B_2 protein.

3.3. Aortic reactivity studies

To test the functional activity of B_2 in aortic rings of B_2^{tg} compared to B_2^n mice endothelium-dependent vasorelaxation, contractile activity in response to phenylephrine, relaxation to the spontaneous NO donor DEA NONOate as well as responses to bradykinin with and without

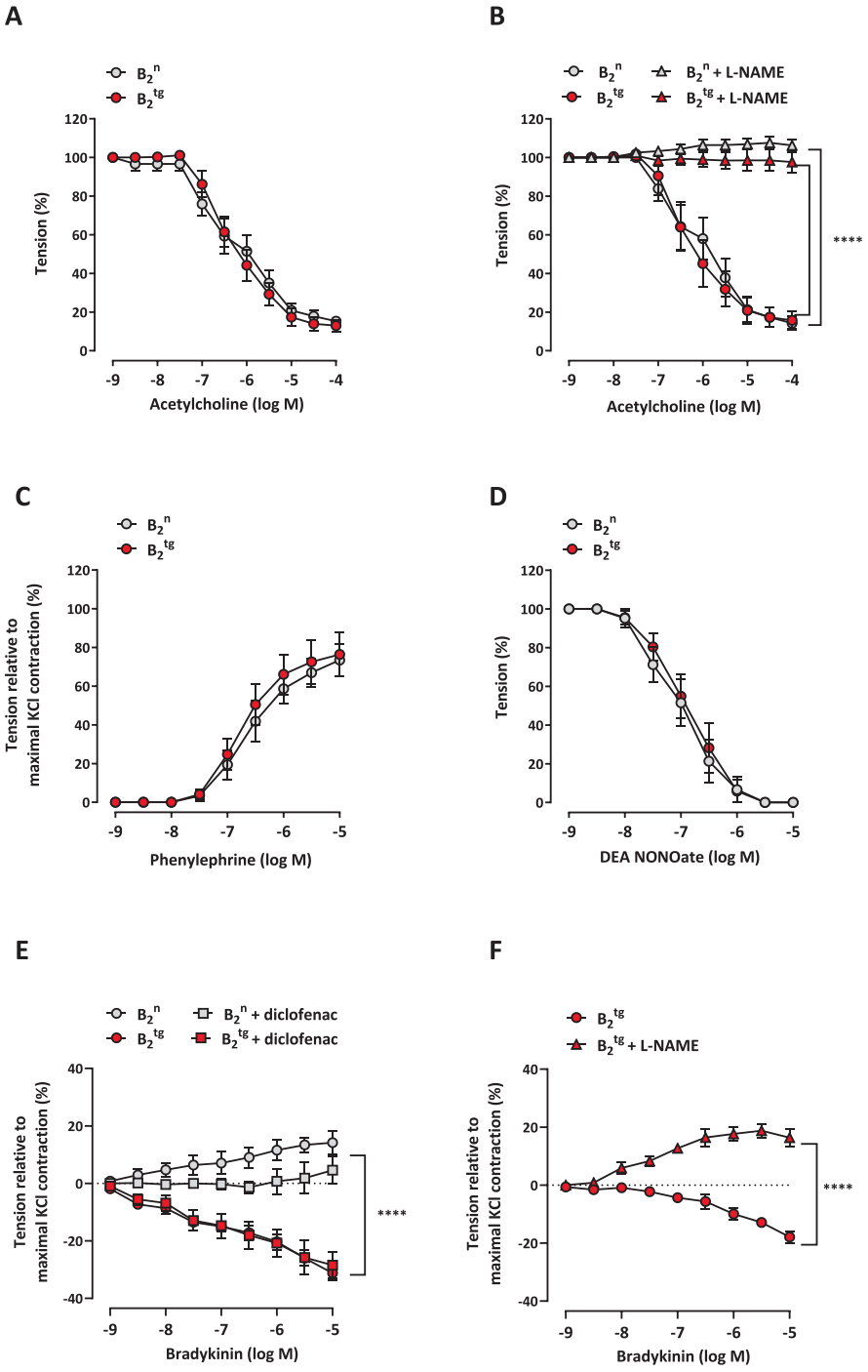

Fig. 3. Aortic reactivity to acetylcholine, phenylephrine, spontaneous NO donor DEA NONOate and bradykinin as obtained by concentration response curves from isolated rings of B_2^{tg} and B_2^n mice. The responses observed were almost identical with **(A)** acetylcholine (n = 8 per group, P = ns) and **(B)** sensitive to the NO-synthase inhibitor L-NAME (n = 5 per group, ****P < 0.0001 vs vehicle), with **(C)** phenylephrine (n = 4 per group, P = ns) as well as **(D)** DEA NONOate (n = 3 per group, P = ns). In contrast, **(E)** bradykinin induced concentration-dependent constriction in aortic rings of B_2^n mice that was abolished by addition of the cyclooxygenase inhibitor diclofenac (n = 4–5 per group, P = ns, B_2^n vs B_2^n plus diclofenac), while vasorelaxation was noted in B_2^{tg} (n = 5–6 per group, ****P < 0.0001 B_2^{tg} vs B_2^n) which was independent of diclofenac (n = 6 per group, P = ns, B_2^{tg} vs B_2^{tg} plus diclofenac). In addition, **(F)** this relaxation was converted by L-NAME to a vasoconstrictor response resembling that in B_2^n and suggesting a NO-dependent counteractivity unmasking bradykinin induced vasoconstriction in B_2^{tg} (n = 8 per group, ****P < 0.0001). Statistical significance was evaluated by two-way repeated measures ANOVA.

preincubation with the cyclooxygenase inhibitor diclofenac or the NO-synthase inhibitor L-NAME were evaluated (Fig. 3).

Endothelium-dependent vasorelaxation to acetylcholine was unaffected by the transgene (Fig. 3**A**) as was its inhibition by L-NAME (Fig. 3**B**). Of note, maximal endothelium-dependent vasorelaxation of aortic rings of B_2^{tg} (87.0 ± 3.0 %) and B_2^{n} mice (84.7 ± 2.3 %) was identical. Likewise, there was no difference between the responses to either phenylephrine (Fig. 3**C**) or DEA NONOate (Fig. 3**D**) suggesting such vascular reactivity remains unchanged. In striking contrast, bradykinin produced vasoconstriction in aortic rings of B_2^{n} that was completely inhibited by diclofenac (Fig. 3**E**), but induced concentration-dependent relaxation in B_2^{tg} independent of diclofenac (Fig. 3**E**). This relaxation was sensitive to L-NAME (Fig. 3**F**) and thus mediated by activation of endothelial NOS. These latter data demonstrate functional activity of the transgene in aortic rings of B_2^{tg} mice.

3.4. Systolic blood pressure and heart rate measurements

To explore systemic vascular effects of the transgene in B_2^{tg} mice, systolic blood pressure and heart rate were measured in conscious mice. Systolic blood pressure was significantly lower in B_2^{tg} than B_2^{n} mice (B_2^{tg}: 125.0 ± 1.6 mmHg; B_2^{n}: 129.9 ± 1.1 mmHg, n = 9 each, P = 0.02, Fig. 4**A**). To verify that the decrease in systolic blood pressure was related to the transgene, the same groups of mice received the specific B_2 antagonist icatibant (i.p.). The systolic blood pressure difference between groups was abolished after icatibant administration (B_2^{n}: 128.6 ± 4.6 vs B_2^{tg}: 128.3 ± 2.0 mmHg, P = ns, Fig. 4**B**), confirming a role of the transgene in systolic blood pressure reduction. In addition, the non-selective inhibitor of cyclooxygenases diclofenac was used to assess whether the decrease in blood pressure observed in B_2^{tg} is, in contrast to aortic reactivity, dependent on the cyclooxygenase-pathway (Fig. 3**E**). Oral administration of diclofenac slightly reduced systolic blood pressure in both B_2^{n} (from 129.9 ± 1.1 into 125.5 ± 2.4 mmHg, n = 9, P = 0.09) and B_2^{tg} (from 125.0 ± 1.6 into 119.8 ± 2.8 mmHg, n = 9, P = 0.12), but there was no statistically significant difference. The percentage change of systolic blood pressure in B_2^{tg} was not significantly different from B_2^{n} (−3.3 ± 1.7 %, n = 9, P = ns), suggesting that systolic blood pressure reduction in B_2^{tg} is most likely not dependent on the cyclooxygenase-pathway. Likewise, treatment with L-NAME had no significant effect on the percentage change of systolic blood pressure in B_2^{tg} (11.3 ± 2.2 %) and in B_2^{n} (7.1 ± 1.3 %, n = 6, P = ns, unpaired *t*-test).

Heart rate was also significantly lower in B_2^{tg} than in B_2^{n} (B_2^{tg}: 567 ±

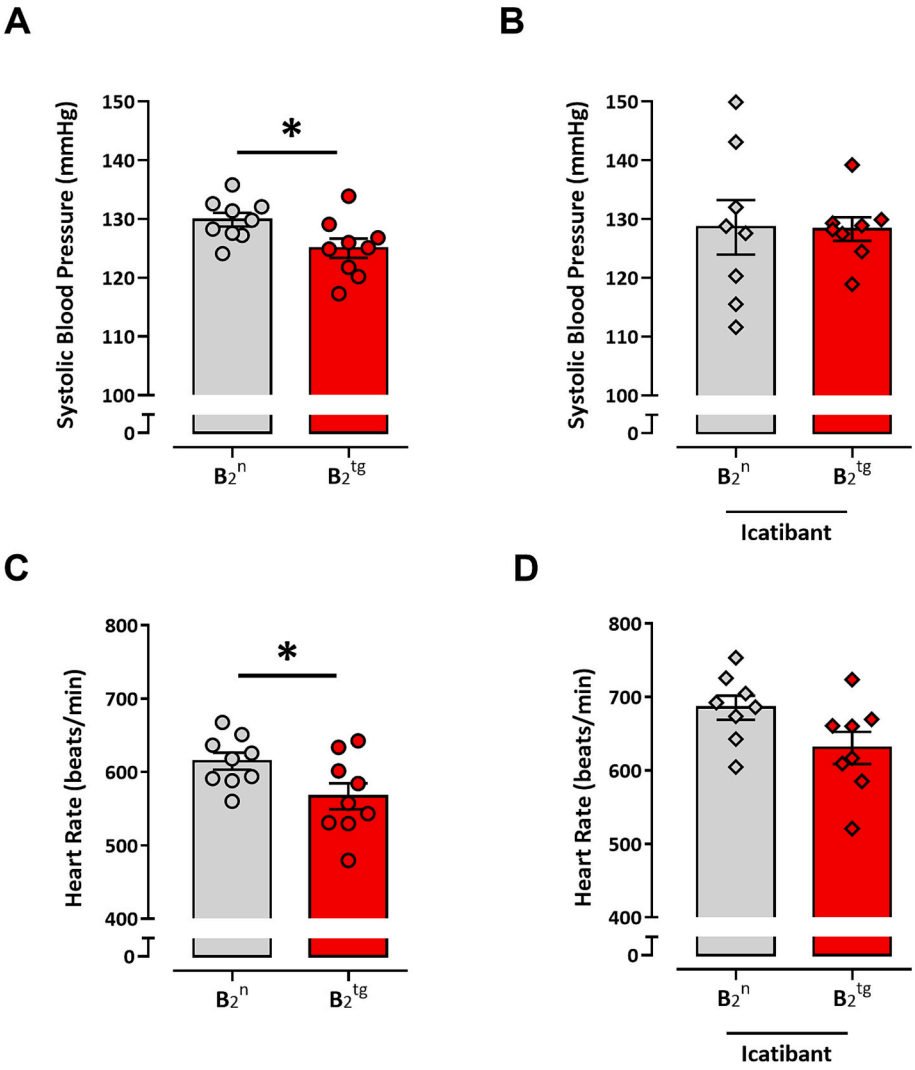

Fig. 4. Systolic blood pressure and heart rate data in conscious mice. (**A**) Systolic blood pressure was significantly lower in B_2^{tg} mice than B_2^{n} (n = 9 each, P = 0.02, unpaired *t*-test). (**B**) The systolic blood pressure difference between groups abolished after prior administration of 1 mg/kg i.p. icatibant indicating dependence on B_2 (n = 8 each, P = ns, unpaired *t*-test). (**C**) Heart rate was significantly lower in B_2^{tg} than in B_2^{n} (n = 9 each, P = 0.03, unpaired *t*-test). (**D**) Icatibant administration abolished the significance of the heart rate difference between B_2^{n} and B_2^{tg} (n = 8 each, P = ns, unpaired *t*-test) but increased heart rate in each line (n = 8–9 each, paired *t*-test, data see Chapter 3.4).

17.7 bpm; B_2^n: 614.7 ± 11.4 bpm, Fig. 4**C**). In addition, administration of icatibant abolished the significant difference (Fig. 4**D**) and thus matched the observations made for systolic blood pressure (Fig. 4**B**). However, icatibant slightly but significantly increased heart rate in both B_2^n (from 614.7 ± 11.4 to 685.5 ± 16.4 bpm, P = 0.02) and B_2^{tg} (from 567 ± 17.7 to 630.8 ± 21.8 bpm, P = 0.049) suggesting that physiologic activation of B_2 exerts a small depression of heart rate in mice. Treatment with L-NAME had similar effect on the percent change of heart rate in B_2^{tg} (−19.1 ± 3.1 %) and B_2^n (–23.4 ± 2.9 %, n = 6, P = ns, unpaired *t*-test). The decrease of heart rate observed in B_2^{tg} in comparison to B_2^n together with the increase in heart rate after icatibant administration suggest at least a minor role of B_2 in murine heart rate regulation.

3.5. Flow-mediated dilation

In vivo vascular functions of B_2^{tg} mice were further explored through FMD analysis (Fig. 5**)**. As expected, the mean flow velocity increased substantially after 20 s of cuff release in both B_2^n (from 54.21 ± 5.79 into 111.75 ± 11.96 mm/sec, n = 7, P = 0.056) and B_2^{tg} (from 53.37 ± 5.81 into 111.12 ± 9.60 mm/sec, n = 9, P = 0.0004). After 3 min, the mean flow velocity values returned nearly to baseline values (B_2^n: 52.51 ± 4.62, B_2^{tg}: 51.27 ± 3.95 mm/sec), **(**Fig. 5**E)**. Importantly, there was no difference in mean flow velocity between B_2^n and B_2^{tg} at all time points (P = ns, Fig. 5**E**). As shown in Fig. 5**F**, no overall improvement of FMD was observed in B_2^{tg} mice (n = 7–9 per group, P = ns vs B_2^n).

3.6. Echocardiography

To explore the effect of endothelial-specific B_2 overexpression on a variety of parameters of cardiac function, non-invasive echocardiography was done in anesthetized B_2^{tg} and B_2^n mice showing no difference in age and body weight (Table 1) and all parameters of heart function in B_2^{tg} were comparable to those in B_2^n. No signs of LV hypertrophy were noticed as the LV dimensions, LV mass and heart weight/BW were comparable. Moreover, parameters of heart diastolic function in B_2^{tg} were similar to those in B_2^n as no significant differences were found in passive early diastolic filling wave/atrial contraction wave (E/A), mitral valve deceleration time (MV DT) and isovolumic relaxation time (IVRT) as shown in Table 1. Collectively these data suggest that overexpression of endothelial B_2 had no effect on cardiac structure and function.

4. Discussion

Infusion of bradykinin in mice [31] and humans [32,33] as well as the effect of captopril in hypertensive patients [34] suggest that activation of B_2 by bradykinin reduces blood pressure. However, in young patients with hereditary angioedema and no overt cardiovascular disease, that is without increasing overall bradykinin concentrations in the

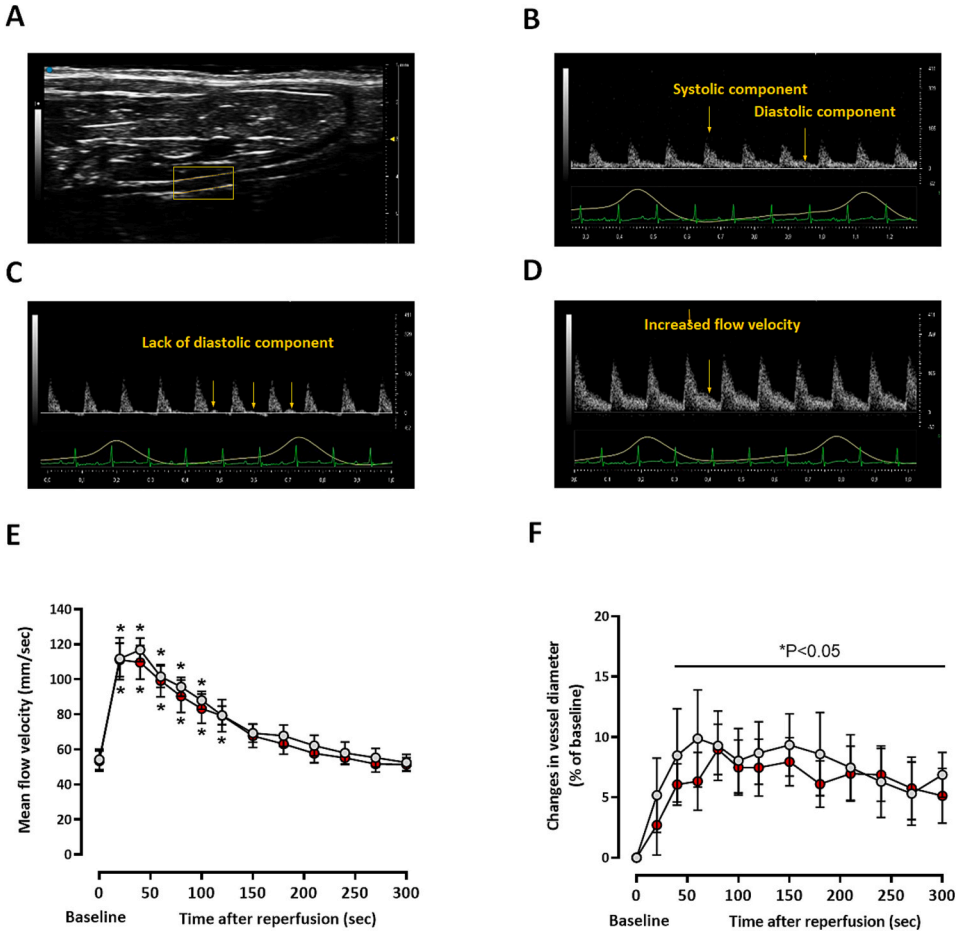

Fig. 5. Flow-mediated dilation (FMD) and mean flow velocity in external iliac artery of B_2^{tg} and B_2^n mice. (**A**) Longitudinal B-mode ultrasound image of external iliac artery and automatic edge-detection (yellow dot-line). Original Doppler flow signal in iliac artery (**B**) at baseline, (**C**) during ischemia and (**D**) 60 s after cuff-release. (**E**) Mean flow velocity over 3 min during reperfusion (i.e. after cuff deflation). No significant difference was shown between B_2^{tg} and B_2^n mice (P = ns, two-way repeated measures ANOVA), but mean flow velocity increased substantially after cuff release at 20, 40, 60, 80, 100, 120 s in B_2^{tg} and B_2^n (*P < 0.05 vs. baseline, using Dunnett's multiple comparisons test following two-way ANOVA). (**F**) Quantification of FMD as percent change of diameter of artery from baseline. No significant difference was found between B_2^{tg} and B_2^n mice (P = ns, two-way repeated measures ANOVA), but mean vessel diameter increased substantially after cuff release at 40 to 300 s in B_2^{tg} and B_2^n (*P < 0.05 vs. baseline, using Dunnett's multiple comparisons test following two-way ANOVA).

Table 1
Echocardiographic parameters and p-values of B_2^{tg} and B_2^n mice.

Parameter	B_2^n, n = 9–10	B_2^{tg}, n = 10	P-value
Age, weeks	12–14	12–14	ns
Weight, g	28.3 ± 0.3	29.3 ± 0.4	ns
Heart Rate, bpm	463.8 ± 13.1	494.6 ± 15.8	ns
End systolic Volume, μL	28.8 ± 1.3	30.4 ± 2.0	ns
End diastolic Volume, μL	62.2 ± 2.1	67.1 ± 4.6	ns
Stroke Volume, μL	33.4 ± 1.2	36.6 ± 2.7	ns
Ejection Fraction, %	53.8 ± 1.2	54.51 ± 0.8	ns
Fractional Shortening, %	10.0 ± 0.6	9.5 ± 1.4	ns
Cardiac Output, ml/min	15.6 ± 0.8	17.9 ± 1.2	ns
LV mass/BW mg/g	3.5 ± 0.1	4.1 ± 0.2	ns
Heart weight/BW mg/g	5.2 ± 0.13	5.1 ± 0.15	ns
LVPW;d, mm/g	0.03 ± 0.0	0.04 ± 0.0	ns
LVPW;s, mm/g	0.03 ± 0.0	0.03 ± 0.0	ns
Cardiac Index, ml/min/g	0.5 ± 0.02	0.6 ± 0.03	ns
IVCT, ms	10.7 ± 0.4	11.5 ± 0.4	ns
IVRT, ms	14.7 ± 0.9	14.5 ± 0.5	ns
E/A	1.3 ± 0.07	1.2 ± 0.05	ns
MV DT, ms	24.72 ± 1.3	26.2 ± 1.6	ns

LV: left ventricular, BW: body weight, LVPW;d: left ventricular posterior wall thickness during diastole, LVPW;s: left ventricular posterior wall thickness during systole, IVRT: isovolumic relaxation time, interval between aortic valve closure and mitral valve opening, IVCT: isovolumic contraction time, interval between mitral valve closure and aortic valve opening, 'E' wave: passive early diastolic filling, 'A' wave: atrial contraction, MV DT: deceleration time of the E wave. Statistical analysis was done using unpaired *t*-test.

systemic circulation, blockade of B_2 by icatibant had no effect on blood pressure [6]. In this report endothelial-specific overexpression of B_2 was associated with only a small but significant reduction in systolic blood pressure and heart rate, while *in vivo* vascular and cardiac functions were not altered by the transgene. These data suggest that endothelial-specific B_2 overexpression has only minor effects on resting circulatory functions *in vivo* in healthy young mice.

Endothelial-specific overexpression of B_2 was shown to be functionally active in small dermal blood vessels and within the circulation including skeletal muscle resistance vessels [4]. The reduction of blood pressure obtained in a newly backcrossed line of this B_2 strain reported here confirms this effect suggesting functionally active overexpression of B_2 in resistance vessels. In the setting of a general overexpression of human B_2 in mice a hypotensive phenotype was reported showing a similar reduction of blood pressure of 13–20 mmHg in two different lines as compared to transgene negative littermates [31]. Therefore, it appears reasonable that the effect on blood pressure following general overexpression was most likely mediated by an increase of endothelial B_2 expression. The effect of global disruption of B_2 on blood pressure at rest are in line with these findings as a significant increase in systolic blood pressure at rest of about 15 mmHg was observed [14]. In contrast, several other studies in B_2 deficient mice showed no change in blood pressure at rest [11–13], but a strong nearly complete inhibition of blood pressure reduction after infusion of bradykinin [13,14]. Hence, the currently available data suggest that physiologic circulating levels of bradykinin have minor effects on B_2 signalling in resistance blood vessels.

In accordance with our findings on blood pressure, *in vivo* evaluation on the reactivity of conductive arteries to increased flow showed just minor variations in B_2^{tg}. Both changes of mean flow velocity and vessel diameter following release of the cuff occlusion showed the expected increase but no difference between the mouse lines. These data suggest that endothelial-specific B_2 overexpression most likely does not contribute to flow-mediated vasodilation and hence does not appear to have any significant impact on the response of conductive blood vessels such as the iliac artery to increased flow induced by reactive hyperaemia. Although kallikrein deficiency cannot be equated with either B_2 deficiency or B_2 overexpression, it was reported that artificially increased flow, instead of reactive hyperaemia, was dependent on both B_2 and tissue kallkrein [35]. However, it has been shown previously that flow-mediated vasodilation of human peripheral conduit arteries *in vivo* is mediated by NO [36] which itself is produced in vascular endothelial cells [37]. Later on, this methodology was adopted to mice and the complete dependence of flow-mediated vasodilation on endothelial NO was confirmed using endothelial NOS (eNOS) knockout mice [38]. Nevertheless, the results of our experiments do not necessarily exclude a role for bradykinin in flow mediated vasodilation as bradykinin might contribute to the dilation of resistance vessels in response to ischemia.

In contrast to the lack of effect of endothelial-specific B_2 overexpression on flow-mediated vasodilation, exogenous bradykinin has been shown to potently dilate dog coronary arteries and human forearm resistance vessels *in vivo* and this is attenuated by inhibition of NOS [32,39]. However, a later study suggested that the underlying mechanism involves rather an endothelium-dependent hyperpolarizing factor and not activation of endothelial NO generation [40]. Early *in vitro* studies revealed that endothelium-dependent vasodilation in response to bradykinin in dog, cat, rabbit and human arteries occurs in an endothelium-dependent manner [41] and that the relaxation of porcine coronary arteries includes activation of eNOS as well as generation of an endothelium-dependent hyperpolarizing factor [42]. A concentration dependent vasoconstriction induced by bradykinin as observed in this study has been previously reported to occur in aortic rings of C57Bl/6 mice [43,44] and of B_2^n as well [4]. This vasoconstriction was blunted by icatibant suggesting B_2 mediated effect and by diclofenac or indomethacin [4,43,44] suggesting that generation of a cyclooxygenase related vasoconstrictor appears to be involved. In striking contrast, bradykinin induced a concentration-dependent relaxation in aortic rings of B_2^{tg}. At first glance, this observation appears paradoxical as an augmentation of vasoconstriction would be expected by overexpression of B_2. However, not only activation of cyclooxygenase but also activation of eNOS is involved following activation of B_2 in aortic rings of mice. Inhibition of eNOS by L-NAME potentiated bradykinin induced vasoconstriction to a similar extend than the aortic constriction to bradykinin that occurs in eNOS deficient mice backcrossed to C57BL/6 [44]. Hence, bradykinin appears to activate eNOS and thereby attenuated vasoconstriction by the cyclooxygenase product. Therefore, two counteracting signal transduction mechanisms are being activated by bradykinin in mouse aortic rings and the net aortic response likely depends on the relative contribution of each of those.

As bradykinin dilated instead of constricted aortic rings of B_2^{tg}, a response that has been observed previously [4], endothelial-specific overexpression of B_2 may have shifted this balance in favour of activation of eNOS. Likewise, overexpression of B_2 in rats using the cadherin 5 promoter resulted in a pronounced relaxation, while in wild-type rats this response was not observed [45]. Indeed, this vasodilator response was not only dependent on intact endothelium and activation of B_2, it was also reversed to vasoconstriction following inhibition of eNOS by L-NAME. A previous report suggested that B_2 and eNOS are physically associated in the endothelial cell membrane and that this vicinity of the two proteins resulted in inhibition of eNOS activity [46]. Once B_2 is being activated it induces a rapid dissociation of this heterodimeric protein complex leading to activation of eNOS presumably because the interaction between B_2 and eNOS blocks flavin to heme electron transfer [47]. Therefore, endothelial-specific overexpression of B_2 may give rise for a larger number of B_2-eNOS complexes which then lead to a sequestration of the total eNOS pool towards B_2 and away from other eNOS inhibiting endothelial proteins such as caveolin [48] or the angiotensin II receptor type 1 [46].

The small but significant decrease of heart rate observed in B_2^{tg} vs. B_2^n was most likely related to increased stimulation of B_2 by endogenous bradykinin as icatibant increased heart rate in both B_2^{tg} and B_2^n and abolished the heart rate difference between groups. Hence, endogenous bradykinin appears to play a role in heart rate regulation by activating B_2. In dogs a more pronounced bradycardic effect was shown after injection of exogenous bradykinin into the left coronary artery [49]. This

effect was reversed to a small increase in heart rate by bilateral vagotomy or by injection of bradykinin into the right atrium. Furthermore, a study in B_2 deficient mice showed a small increase in heart rate which became stronger with increasing age and remained stable beyond 6 months [50]. Likewise, another study in B_2 deficient mice reported a sustained but still small increase in heart rate in mice 8–12 months of age [20]. In both studies the extent of the heart rate elevation was similar to the heart rate reduction observed in this study with B_2^{tg}. However, other studies in B_2 deficient mice reported no change of heart rate [11] or even a decrease [51]. Although the reports in different strains of B_2 deficient mice show somehow contradictory results, the findings of this study obtained with another mouse model support a small but significant role of endothelial-specific B2 overexpression on heart rate regulation.

Other studies explored the possible mechanisms underlying bradykinin-mediated decrease in heart rate, which could be mediated by cardiac vagal tone modulation. The first findings were reported from a study in adult mongrel dogs bilaterally vagotomized at the cervical level. Following pre-treatment with propranolol to exclude any baroreceptors mediated effects, intracoronary injection of bradykinin resulted in a significant reduction of heart rate [52]. This reduction was potentiated by concomitant treatment with captopril and completely inhibited by icatibant. Although the vagotomy procedure may suggest that bradykinin does not work by increasing vagal stimulation one has to bear in mind that vagotomy at the cervical level still leaves intact parasympathomimetic neurons in the heart. Indeed, later findings from studies on isolated autonomically decentralized hearts (Langendorff preparation) from rabbits investigating the mechanism of the negative chronotropic action of bradykinin provided evidence for a parasympathomimetic mechanism. The results of this study using co-infusion with bradykinin and hexamethonium or atropine suggested that bradykinin exerts its negative chronotropic action by activation of intrinsic cholinergic neurons [53]. In addition, this same activity played a role in reversing tachycardia induced by catecholamines such as isoproterenol and dobutamine. In line with these findings, stimulation of B_2 in neonatal rat nucleus ambiguous neurons produced a rise of intracellular Ca^{2+} concentrations mediated by inositol 1,4,5-trisphosphate and ryanodine receptors indicative of G-protein subunit q coupling of B_2 [54]. Of note, the nucleus ambiguous includes preganglionic parasympathetic neurons which innervates postganglionic parasympathetic neurons in the heart and *in vivo* microinjection of bradykinin in this region induced bradycardia inhibited by icatibant. The results of these studies are in line with our finding that endothelial overexpression of B_2 directly affect the heart rate and manifested as bradycardia in B_2^{tg}.

Although some studies reported that icatibant did not affect heart rate [55,56], our results showed that icatibant increased heart rate in mice regardless B_2 overexpression. In agreement with this, i.v. icatibant administration in conscious rats and intra-arterial icatibant administration in conscious spontaneously hypertensive rats showed an increase in heart rate [57,58]. In humans, i.v. icatibant infusion for 6 h enhanced the increase in heart rate that was already observed after 1 month administration of angiotensin II type 1 receptor antagonist valsartan [59]. The discrepancy on the effect of icatibant on heart rate may be related to differences in routes of administration, doses, use of anaesthesia, and the studied species. Therefore, the potential role of B_2 in heart rate regulation should not be rejected. On the other hand, echocardiographic findings in B_2^{tg} did not show any variation of several cardiac function parameters leaving heart rate reduction as the only significant alteration induced by endogenous bradykinin.

As heart rate and systemic vascular resistance largely determines mean arterial blood pressure, any change of one parameter is expected to impact the other. Hence, the observed reduction of blood pressure in B_2^{tg} might be the result of both, decreased vascular resistance or decreased heart rate. It is difficult to distinguish this in our study, as we did not investigate the reactivity of bradykinin in isolated resistance blood vessels. Moreover, it was demonstrated that in contrast to blockade of ß-adrenergic receptors selective pharmacological reduction of heart rate by ivabradine does not change blood pressure, neither in mice [60,61] nor in humans [62]. In view of the proposed mechanism of action underlying bradycardia induced by bradykinin one must keep in mind that activation of parasympathomimetic signalling reduces blood pressure by opposing the effects of adrenergic signals. To this end, it appears likely that shifting the balance of the autonomic nervous system activity more in direction of parasympathetic branch may best explain the moderate reduction of blood pressure in B_2^{tg}.

Our study has several limitations. The level of endogenous bradykinin was not measured in this study as the accurate quantification of bradykinin levels in mice is merely impossible, despite published improvement in analytical accuracy in human blood [63]. Hence, we could not exactly define whether levels of bradykinin under resting conditions might be different between B_2 overexpression mice and controls. Nevertheless, activation of B_2 and subsequent changes of tissue responses are mediated by bradykinin as proven by inhibition with icatibant in many experimental studies [3,4,64–66], and in hereditary [6] and ACE inhibitor-induced angiodema [9]. Likewise, another newly developed B_2 receptor antagonist, deucrictibant, showed clinical efficacy in hereditary angioedema [67] and recent evaluation of clinical trials and guidelines on treatment of hereditary angioedema did not mention any data or doubt that endogenous bradykinin is the critical trigger [68]. In addition, an intrinsic activation of B_2 has not been reported yet, except in one cell culture study with transfected B_2 [69]. Finally, our results cannot be extrapolated to comorbidities such as thrombotic diseases like stroke or myocardial infarction or conditions which are well known risk factors, for example hypertension, hyperglycemia or overt diabetes. From a clinical perspective, further research is needed to explore the long-term safety of continuous changes of endogenous bradykinin levels by certain therapeutic agents in cardiovascular comorbidities.

In summary, endothelial-specific B_2 overexpression induced mild hypotension and bradycardia while having no effect on flow-mediated dilation or several cardiac function parameters. These minor effects of endogenous bradykinin within the cardiovascular system in healthy young mice suggest that even live-long downregulation of endogenous generation of bradykinin, an approach that shows beneficial effects in hereditary angioedema, may not be associated with derangements of cardiovascular functions. The long-term safety of continuous changes in bradykinin levels and endothelial B_2 signalling as well as safety of icatibant or new orally active B_2 antagonists may be of concern in patients with cardiovascular comorbidities and deserves further rigorous clinical investigation.

CRediT authorship contribution statement

Sara Metry: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Tatsiana Suvorava:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jens W. Fischer:** Resources. **Vu Thao-Vi Dao:** Writing – review & editing, Methodology. **Georg Kojda:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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