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RESEARCH PAPER

The role of bradykinin receptor type 2 in spontaneous extravasation in mice skin: implications for non-allergic angio-oedema

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BACKGROUND AND PURPOSE

Non-allergic angio-oedema is a life-threatening disease mediated by activation of bradykinin type 2 receptors (B_2 receptors). The aim of this study was to investigate whether activation of B_2 receptors by endogenous bradykinin contributes to physiological extravasation. This may shed new light on the assumption that treatment with an angiotensin converting enzyme inhibitor (ACEi) results in an alteration in the vascular barrier function predisposing to non-allergic angio-oedema.

EXPERIMENTAL APPROACH

We generated a new transgenic mouse model characterized by endothelium-specific overexpression of the B_2 receptor ($B2^{tg}$) and established a non-invasive two-photon laser microscopy approach to measure the kinetics of spontaneous extravasation *in vivo*. The $B2^{tg}$ mice showed normal morphology and litter size as compared with their transgene-negative littermates ($B2^{n}$).

KEY RESULTS

Overexpression of B_2 receptors was functional in conductance vessels and resistance vessels as evidenced by B_2 receptor-mediated aortic dilation to bradykinin in presence of non-specific COX inhibitor diclofenac and by significant hypotension in $B2^{tg}$ respectively. Measurement of dermal extravasation by Miles assay showed that bradykinin induced extravasation was significantly increased in $B2^{tg}$ as compared with $B2^{n}$. However, neither endothelial overexpression of B_2 receptors nor treatment with the ACEi moexipril or B_2 antagonist icatibant had any effect on spontaneous extravasation measured by two-photon laser microscopy.

CONCLUSIONS AND IMPLICATIONS

Activation of B_2 receptors does not appear to be involved in spontaneous extravasation. Therefore, the assumption that treatment with an ACEi results in an alteration in the physiological vascular barrier function predisposing to non-allergic angio-oedema is not supported by our findings.

Abbreviations

B_2 receptor, bradykinin type 2 receptor; EP receptor, E-prostaglandin receptor; hprt1, hypoxanthine 8 guanine phosphoribosyl transferase; L-NAME, L-nitroarginine-methyl-ester; $B2^{tg}$, mouse model characterized by endothelium-specific overexpression of B_2 receptors; L-NA, N^G-nitro-l-arginine; RAAS, renin angiotensin aldosterone system; $B2^{n}$, transgene-negative littermates of $B2^{tg}$

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Introduction

Non-allergic angio-oedema is a well-known side effect of angiotensin converting enzyme inhibitors (ACEi), for example, captopril, as well as blockers of angiotensin-II-type-1-receptors (AT_1 receptors) such as losartan (Bas *et al.*, 2007). These drugs are used worldwide to treat many million patients with hypertension, heart failure with reduced ejection fraction, left ventricular dysfunction or diabetes including nephropathy or stroke, because pharmacological inhibition of the renin angiotensin aldosterone system (RAAS) confers organ protection and reduces morbidity and mortality (Schmieder *et al.*, 2007). The most frequent adverse events among patients unable to tolerate ACEi are cough (88.2%), followed by symptomatic hypotension (4.1%), non-allergic angio-oedema, anaphylaxis (1.3%) or renal dysfunction (1%) (Yusuf *et al.*, 2008a).

Non-allergic angio-oedema induced by RAAS blockers is often overlooked or misdiagnosed, although it may develop into a serious life-threatening condition, for example, if swelling occurs at the larynx (Bas *et al.*, 2007). Non-allergic angio-oedema occurrs at frequencies of about 0.5% with ACEi and about 0.25% with valsartan (Yusuf *et al.*, 2000; Pfeffer *et al.*, 2003; Yusuf *et al.*, 2008b). Furthermore, the new drug combination sacubitril/valsartan (McMurray *et al.*, 2014) has the potential to induce non-allergic angio-oedema (Bas *et al.*, 2015a) and a life-threatening case has been reported recently (Raheja *et al.*, 2017). Although intestinal manifestations of non-allergic angio-oedema caused by ACEi have been described (Palmquist and Mathews, 2017), almost all cases induced by RAAS blockers are localized to the upper aerodigestive tract. Such swellings often require emergency treatment, and fatal cases have been reported (Ulmer and Garvey, 1992; Oike *et al.*, 1993; Dean *et al.*, 2001; Atalay *et al.*, 2015). In view of the incidence of RAAS-blocker induced non-allergic angio-oedema and the frequent use of such drugs, about 35 000 cases are expected to occur in Germany each year. Unfortunately, there is no evidence-based diagnosis (Bas *et al.*, 2010), presumably leaving many cases unrecognized.

A direct and accurate measurement of plasma **bradykinin** concentrations is technically very difficult, and this is an important reason for the lack of an accurate diagnosis of non-allergic angio-oedema. Firstly, the vein puncture necessary for blood collection will induce contact activation *via* factor XII and hence activation of kallikrein and generation of bradykinin (Kaplan and Joseph, 2014). Secondly, bradykinin is degraded by several proteases including aminopeptidase P, neutral endopeptidase, carboxypeptidase N and dipeptidylpeptidase IV, and the activity of these proteases during blood collections is difficult to control, and thirdly, the concentration of bradykinin in the circulation is very low (Nussberger *et al.*, 1998; Nussberger *et al.*, 2002). In addition, one needs to consider that local concentrations of bradykinin in angio-oedema tissue might be substantially different from concentrations in venous blood drawn from the antecubital vein (Nussberger *et al.*, 1999).

Methods

Animals

C57Bl/6 mice (male, 3–4 months old, 24–28 g) were purchased from JANVIER LABS (Le Genest-Saint-Isle, France). We cloned a plasmid consisting of pBluescript II SK+ -Vector (Stratagene, Amsterdam), the Tie-2-promoter (2.1 Kb), the human bradykinin type 2 receptor (**B_2 receptor**) cDNA (1.3 Kb), the SV40 poly-A-signal and a Tie-2 intron fragment, designated as Tie-2-enhancer (10 Kb). This plasmid was based on plasmids cloned previously to generate other transgenic mice (Lauer *et al.*, 2005; Suvorava *et al.*, 2005) in which endothelial-specific overexpression of the target protein was proven by confocal microscopy (Oppermann *et al.*, 2011; Suvorava *et al.*, 2017). In cooperation with Oliver Lieven and Ulrich Rüther (Institute for Animal Developmental and Molecular Biology, Heinrich-Heine-University), the construct was microinjected into fertilized F_2-eggs of F_1 (C57BL/6 × C3H/He) mice. Microinjected eggs were transferred into the oviducts of pseudo-pregnant mice and allowed to develop to term. The transgenic B_2 receptor mouse line ($B2^{tg}$) including their transgenic negative littermates ($B2^{n}$) was established and backcrossed to C57BL/6 for more than 10 times. Transgenic mice were bred and housed in the animal facility (ZETT, UKD Düsseldorf, Germany), and male, 3–4 months old (24–28 g) mice including C57BL/6 were used for experiments. Animals (n = 3–5 per cage) were housed in standard cages, kept in a 12-h light/dark circle and received a standard mouse chow and acidified water (pH = 3–4) *ad libitum*. To avoid the possible influence of the oestrous cycle on skin permeability, we chose to investigate male mice only. The experiments were performed according to the guidelines for the use of experimental animals, as given by the German 'Tierschutzgesetz' (approval references: 8.87-50.10.34.08.223, 8.87-51.04.20.09.383, 84-02.04.2012.A194, 84-02.04.2016.A114) and the 'Guide for the Care and Use of Laboratory Animals' of the US National Institutes of Health. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Randomization and blinding

In the experiments with mice, comparisons were made between either $B2^{n}$ and $B2^{tg}$ or $B2^{tg}$ and C57BL/6 so that randomization was not possible. In experiments where C57BL/6 were treated with different drugs, mice were randomly selected using cage numbers. Blinding was not performed, but experiments were done by different authors at different time points (please refer to section Authors contributions).

Quantitative real-time PCR experiments

Total RNA from mouse lung tissue was isolated using the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany), which included an on-column DNAse digestion. cDNA was synthesized from 1 μg total RNA by Turbo™ DNase (Ambion, Carlsbad, USA) according to the manufacturer's protocol. Quantitative real-time PCR experiments were performed with TaqMan® Gene Expression Assays (Applied Biosystems, Weiterstadt, Germany) using Mm01339907_m1 and Hs00176121_m1 to detect mouse and human B_2 receptors respectively. Co-amplification of hypoxanthine guanine phosphoribosyl transferase (hprt1) cDNA was performed for normalization purposes with the TaqMan Gene Expression Assay for mouse hprt1 (Mm00446968) and human hprt1 (Hs02800695). Quantitative real-time PCR experiments were carried out on a 7300 Real-Time PCR System (Applied Biosystems, Weiterstadt Germany). B_2 receptor mRNA

expression levels relative to hprt1 were determined using the $^{\Delta\Delta}$Ct method relative to paired controls. As for transgene human B_2 receptors, individual data points were calculated.

Western blot

We have evaluated the specificity of several antibodies directed against the B_2 receptor and found that a rabbit monoclonal anti-B_2 receptor antibody (Abcam, Cambridge, UK) appears to be reliable (Khosravani *et al.*, 2015), that is, there was just a faint staining in lung tissue of B_2 receptor$^{-/-}$ mice. Briefly, a total of 50–100 mg protein was loaded onto 10% SDS-PAGE gels. Blotting on nitrocellulose membranes was followed by incubation with the anti-B_2 receptor antibody and a mouse monoclonal antibody detecting GAPDH (1:5000, Sigma, Munich, Germany) for loading control. Finally, fluorescent detection was achieved by labelling with either IRDye 680LT goat anti-mouse IgG or IRDye 800CW goat anti-rabbit IgG (both LI-COR Biosciences, Bad Homburg, Germany) using an Odyssey Infrared Imager 9120 (LI-COR Biosciences GmbH, Bad Homburg, Germany).

Organ harvest and lung water content

Mice were killed by inhalation of carbon dioxide, and body weight, left lung weight and heart weight were measured. To evaluate lung water content, whole left lung wet weight was first determined. The lung tissue was then dried at 58 °C in a drying oven to a constant weight, and the dry weight of lung tissue was determined. The relative water content of lung tissue was calculated using the following equation: lung water content = (lung wet weight – lung dry weight)/lung wet weight × 100%.

Aortic reactivity

Aortic reactivity to bradykinin was studied in aortas of B2^{tg} and B2^{n} by cumulative application of bradykinin (0.01-nmol·L^{-1} to 0.1 mmol·L^{-1}) after submaximal pre-contraction with 0.2 μmol·L^{-1} phenylephrine as described previously (Khosravani *et al.*, 2015). To evaluate endothelium-dependent part of the aortic reaction to BK, aortic responses to increasing concentrations of BK were assessed in the endothelium-intact and endothelium-denuded aortic segments. The endothelium was removed by gently rubbing the intimal surface of the aortic ring with a small wooden stick. In addition, the non-selective **COX1/COX2** inhibitor **diclofenac** (10 μmol·L^{-1}) alone, diclofenac and the selective antagonist of B_2 receptors **icatibant** or diclofenac (10 μmol·L^{-1} each) and the NOS inhibitor **L-nitroarginine-methyl-ester (L-NAME**, 100 μmol·L^{-1}) were administered 30 min before the application of BK. In all organ bath studies, only one BK concentration-response curve per aortic ring was recorded and analysed. Data given are normalized to the maximal constriction obtained with 80 mmol·L^{-1} to control for different sizes and muscle content of aortic rings.

Measurement of blood pressure and heart rate

Systolic blood pressure and heart rate were measured in awake 3–4 month-old male B2^{tg} and B2^{n} mice using an automated tail cuff system (Visitech Systems, Apex, North Carolina) as described previously (Kojda *et al.*, 1999). In each experiment, an adaptation period lasting 7–10 days preceded counted measurements. In each mouse, recordings were made following three consecutive rounds of 10 separate measurements each day, and measurements for a total of 7 days were summarized to obtain the systolic blood pressure values for each mouse. In some experiments, mice were treated p.o. with N^G-nitro-l-arginine (L-NA, 100 mg·kg^{-1}) for 7 days followed by combined treatment with L-NA (100 mg·kg^{-1}) and diclofenac (10 mg·kg^{-1}) for another 4 days. The drinking water was replaced daily.

Miles assay

We used this assay to quantify dermal extravasation of the albumin-bound dye **Evans blue** as initially described by Miles and Miles (1952). Briefly, mice were anaesthetized using 100 mg·kg^{-1} ketamine and 5 mg·kg^{-1} xylazine and received an i.v. bolus of the dye (30 μmol·kg^{-1}). The dorsal skin was shaved completely, and 30 μL of vehicle (PBS), bradykinin, **labradimil** and **histamine** were applied by i.d. injection. The highest concentration translates to a total amount of 2-nmol of bradykinin per injection. In some experiments, mice received 0.3 mg·kg^{-1} **moexipril** (Sigma-Aldrich, Munich, Germany) or 2 or 20 nmol icatibant 2 h prior to Evans blue by i.v. or i.p. injection. A dose–response curve for bradykinin was obtained by simultaneous i.d. injections at different spots on the dorsal skin of the same mouse. In other experiments, bradykinin was injected repeatedly, that is, 10 and 20 min after the first injection in order to obtain a time-dependency. After 30 min, the still anaesthetized mice were killed by cervical dislocation. The skin was removed, and the circular blue dots were cut out with a special circular cutter so that each tissue specimen had the same diameter. The wet weight of the skin specimens was determined. The dye was eluted in 1 mL N,N-dimethylformamide (Sigma-Aldrich, Munich, Germany) for 24 h and quantified at a wavelength of 620 nm using a spectrophotometer (Beckman Instruments GmbH, Munich, Germany). The concentration determined was related to the wet weight and expressed in μg dye g^{-1} tissue. In each experiment, the value obtained in the vehicle-treated tissue was set to 1, and all other values were related to the vehicle value. A non-injected skin tissue specimen was used as an additional control.

Two-photon laser microscopy

We established a two-photon laser methodology allowing us to quantify endothelial permeability in non-inflammatory conditions, that is, spontaneous permeability. The procedure was performed under anaesthesia (using 100 mg·kg^{-1} ketamine and 5 mg·kg^{-1} xylazine) in C57BL/6 mice, and in B2^{tg} mice and their transgene negative littermates B2^{n}. One of the ear lobes was positioned under the microscope, and a randomly selected location within the inner part of the ear was imaged using an Olympus FV1000MPE two-photon laser scanning microscope (Tokyo, Japan) equipped with a 25X, NA1.05 water dipping objective and a fs-pulsed, mode-locked MaiTai DeepSee Ti:Sapphire laser (Spectra-Physics, Mountain View, CA, USA) tuned to 800 nm. Fluorescence was detected by photo multiplier tubes with corresponding bandpass filters and colour-coded according to matching emission wavelength of the fluorescent dyes used. Mice received two different fluorescent dyes of different molecular weight by i.v. injection, a green fluorescent dye that cannot leave the endothelial lumen (Fluorescein Isothiocyanate-Dextran

250 kD, Sigma-Aldrich, Munich, Germany, 1.5 mg in 50 μL per mouse) and a red fluorescent dye that easily traverses the endothelial barrier over time (Alexa Fluor® 594-dextran 10 kD, Fisher Scientific, Schwerte, Germany, 250 μg in 50 μL per mouse). Blood vessels were visualized using the green fluorescent signal and used to adjust and focus the microscope. To visualize the extravasation, kinetic time series of subsequent image stacks were recorded every 30 s over 1 h after injection of the red labelled fluorescent dye. Image stacks of 8 xy-sections (1024 × 1024 pixels, view field 509 × 509 μm) were obtained at successive 3-μm-depth positions (z = 24 μm). 3D reconstructions and image analyses were performed using Imaris Software (Bitplane, Zürich, Switzerland). In some experiments, mice received 0.3 mg·kg^{-1} moexipril (Sigma-Aldrich, Munich, Germany) or 2 nmol icatibant prior to the procedure by i.p. injection. The still anaesthetized mice were killed by cervical dislocation at the end of the experiment.

Statistics

All data were analysed by a standard computer programme (GraphPad Prism PC software, version 6.04) and are expressed as mean ± SEM of n individual samples as stated in Results and Figure Legends. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Statistical calculations for each experiment are based on the number of animals as stated in Results and Legends. Data sets were tested for Gaussian distribution using D'Agostino & Pearson omnibus normality test, Shapiro–Wilk normality test or KS normality test. Scatter plots or before-after charts did not reveal unusual or interesting aspects of the data not obvious from the bar charts. Statistical comparisons between groups were performed by either Student's t-tests or Tukey's multiple comparison *post hoc* test following one-way ANOVA (for more than two groups). To compare the time-course of three or more treatment groups, two-way ANOVA was used. In this case, Sidak multiple comparison *post hoc* test was applied to evaluate significant differences. *Post hoc* tests were run only if F achieved $P < 0.05$, and there was no significant variance inhomogeneity. $P < 0.05$ was considered statistically significant.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

Characterization of $B2^{tg}$ mice

Quantitative real-time PCR with lung mRNA using primers specific for the human or the mouse B_2 receptor cDNA revealed a strong expression of human B_2 receptor in $B2^{tg}$ mice, while no expression of human B_2 receptors was detectable in transgene negative littermates B^n (Figure 1A). Furthermore, expression of the human B_2 receptor did not alter the expression of murine B_2 receptors in $B2^{tg}$ mice indicating that activation of B_2 receptors by bradykinin has no effect on B_2 receptor expression as described previously (Khosravani *et al.*, 2015). Surprisingly, initial Western blot analysis of lung tissues showed no difference in signals between $B2^{tg}$ and $B2^n$ mice. A similar result was obtained in a second series of Western blots investigating lung tissue (Figure 1B). However, in aortic tissue consisting of a lot less cell types and proteins, we detected a second band just a little below the murine B_2 receptor signals, which was observed exclusively in $B2^{tg}$ mice (Figure 1B, C). This observation suggests that the detection of the human B_2 receptor protein was masked in lung tissue (Figure 1B, D).

To investigate whether overexpression of B_2 receptors may promote plasma extravasation, organ wet weight was determined but there was no difference observed (Table 1). Likewise, there was no difference in the percentage of lung and heart weight loss between $B2^n$ and $B2^{tg}$ following drying of the tissue specimens suggesting that lung and heart water content was identical.

To assess whether the function of the B_2 receptor is increased in $B2^{tg}$ in conductance vessels, we used aortic ring preparations in organ bath studies. Previous experiments in C57BL/6 have shown that bradykinin induced concentration-dependent constrictions of aortic rings that were slightly pre-constricted with 0.2 μmol·L^{-1} of phenylephrine (Khosravani *et al.*, 2015). Removal of the endothelium strongly reduced the maximal constriction, while treatment with L-NAME increased the contractile response. The contraction due to bradykinin was completely inhibited by icatibant and partially inhibited by diclofenac indicating that it is mediated by activation of endothelial B_2 receptors and dependent on COX activity (Khosravani *et al.*, 2015). As shown in Figure 2A, we also observed that diclofenac significantly reduced aortic constrictions to increasing concentrations of bradykinin in aortic rings of $B2^{tg}$ mice. In striking contrast to their transgene negative littermates $B2^n$, we found a significant aortic dilation in $B2^{tg}$ following pre-incubation with diclofenac (Figure 2B) that was completely inhibited by endothelial denudation, the NOS inhibitor L-NAME and icatibant. These data indicate that B_2 receptor overexpression is functionally active in conductance vessels of $B2^{tg}$. Likewise, functionally active generation of NO in response to bradykinin occurs in aortic rings of $B2^n$ and $B2^{tg}$ mice.

As shown in Figure 2C, overexpression of the B_2 receptor transgene resulted in a significant reduction in systolic blood pressure in conscious mice. To evaluate whether overexpression of B_2 receptors changes the bioavailability of endogenous NO, $B2^{tg}$ and $B2^n$ mice were treated with the NO-synthase inhibitor L-NA for 7 days. As expected, L-NA elevated blood pressure significantly in $B2^n$ and in $B2^{tg}$. However, the percentage increase was not different between the strains as the 16.9 ± 2.9% increase in blood pressure observed in $B2^n$ mice was not significantly different to the 12.5 ± 2.8% increase of blood pressure in $B2^{tg}$ (n = 6 each, Student's t-test). Addition of diclofenac into the L-NA drinking water for 4 days did not significantly change systolic blood pressure in both strains. In contrast, the transgene did not change heart rate (Figure 2D). Likewise, there were no differences in heart rate between the strains

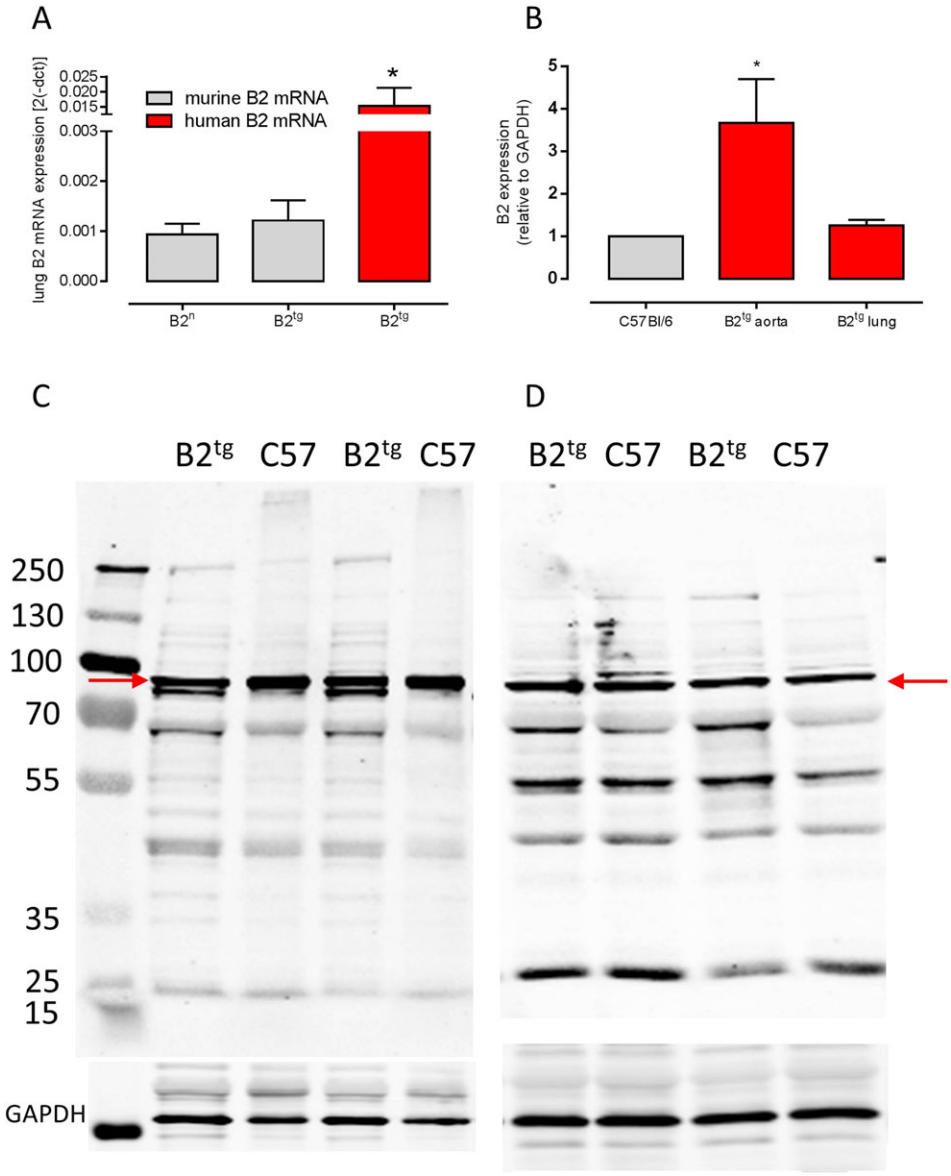

Figure 1

(A) Expression of murine and human B_2 receptor (B2) mRNA isolated from lung tissues of $B2^{tg}$ and their transgene negative littermates $B2^{n}$. There was a strong expression of human B_2 receptor mRNA in $B2^{tg}$, whereas no signals were seen in $B2^{n}$. Despite the 16.4-fold stronger expression of human B_2 receptor mRNA, there was no difference in murine B_2 receptor mRNA between $B2^{n}$ and $B2^{tg}$ (n.s., one-way ANOVA, $*P < 0.05$ vs. murine B_2 receptor mRNA, Tukey's multiple comparison test). (B) B_2 receptor protein expression in aortic and lung tissue of $B2^{tg}$ as compared with C57BL/6. Surprisingly, initial Western blot analysis of lung tissues showed no difference in the signals between $B2^{tg}$ and $B2^{n}$. A similar result was obtained in a second series of Western blots investigating lung tissue ($n = 6$, one-way ANOVA, n.s. vs. C57BL/6). However, in aortic tissue, we detected a second band just a little below the murine B_2 receptor signals, which was observed exclusively in $B2^{tg}$ ($n = 5$, one-way ANOVA, $*P < 0.05$ vs. C57BL/6, Tukey's multiple comparison test). (C) Examples of a full blot showing B_2 receptor protein expression in aortic tissue (ladder shown left in kDa) and (D) in lung tissue (red arrows indicate B_2 receptor signals, signals of GAPDH were detected at 36 kDa with a different secondary antibody and are shown separately).

after treatment with L-NA or L-NA plus diclofenac, although these treatments resulted in a significant reduction in heart rate, as reported previously in mice (Kojda *et al.*, 1999) and rats (Müller-Strahl *et al.*, 2000).

Miles assay

We used the Miles assay to quantify dermal extravasation of the albumin-bound dye Evans blue. Figure 3A illustrates the dots developing during 30 min at the injection site and

Table 1

Morphological data of $B2^{tg}$ as compared with their transgene negative littermates $B2^{n}$

Parameter (mean ± SEM)	$B2^{n}$	$B2^{tg}$
Body weight (BW), g	30.2 ± 1.1	29.5 ± 0.7
Left ventricular myocardium weight (LVW), mg	110.7 ± 4.7	102.9 ± 4.0
LVW/BW, $mg \cdot g^{-1}$	3.5 ± 0.1	3.6 ± 0.1
Lung weight, mg	162.5 ± 7.0	182 ± 6.7
Lung water content, %	79.7 ± 1.9	81.7 ± 2.2
Heart weight, mg	151.5 ± 4.5	149.9 ± 3.1
Heart water content, %	77.5 ± 2.2	72.4 ± 3.5

P-values were determined using Student's unpaired *t*-test.

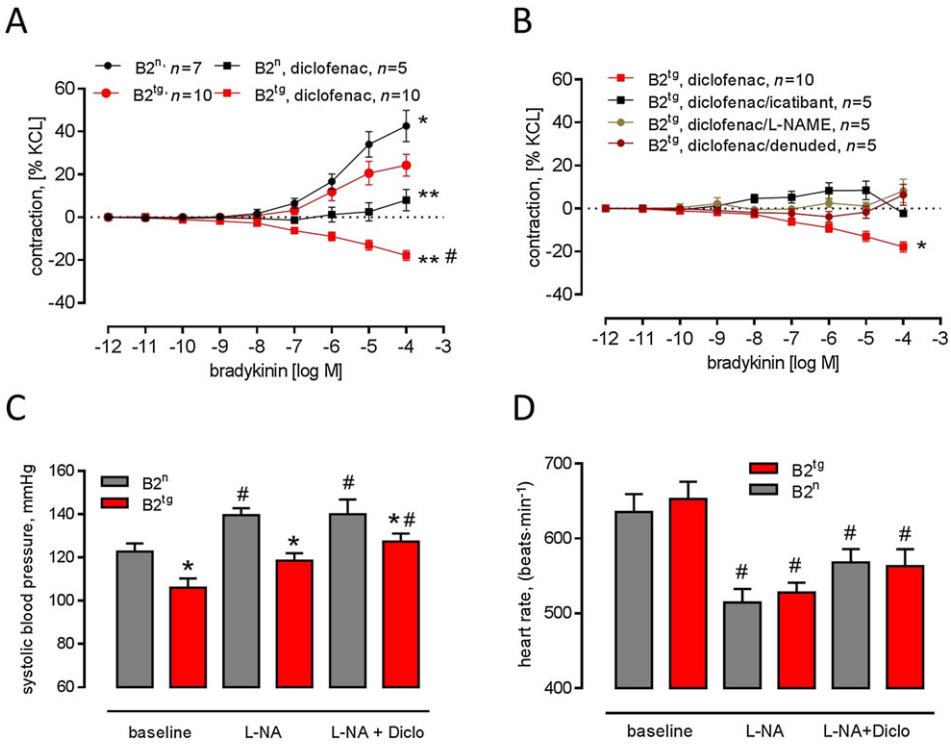

Figure 2

Reactivity to increasing concentrations of bradykinin of mouse aortic ring preparations slightly preconstricted with phenylephrine (0.2 $\mu mol \cdot L^{-1}$). (A) Bradykinin induced an increase in vascular tone that was significantly stronger in $B2^{n}$ ($P < 0.05$, two-way ANOVA, $^{*}P < 0.05$ Sidak multiple comparison test) and significantly reduced by pre-incubation with 10 $\mu mol \cdot L^{-1}$ diclofenac ($^{**}P < 0.05$ Sidak multiple comparison test). In $B2^{tg}$, a vasodilatation was observed in the presence of diclofenac showing a significant difference from that in $B2^{n}$ ($^{\#}P < 0.05$ Sidak multiple comparison test). (B) This vasodilatation was abolished by endothelial denudation, pre-incubation with 10 $\mu mol \cdot L^{-1}$ icatibant or 100 $\mu mol \cdot L^{-1}$ L-NAME ($P < 0.05$ two-way ANOVA, $^{*}P < 0.05$ vs. pretreatment or endothelial denudation, Sidak multiple comparison test). (C) Overexpression of the B_2 receptor transgene resulted in a significant reduction of systolic blood pressure in conscious mice ($n = 6$ each, $P < 0.05$, one-way ANOVA, $^{*}P < 0.05$ Tukey's multiple comparison test). Oral treatment with the NO-synthase inhibitor L-NA (100 $mg \cdot kg^{-1}$) for 7 days significantly elevated systolic blood pressure ($n = 6$ each, $^{\#}P < 0.05$ Tukey's multiple comparison test following one-way ANOVA) but the difference between $B2^{n}$ and $B2^{tg}$ remained. Additional oral treatment with diclofenac (10 $mg \cdot kg^{-1}$) for another 4 days had no further effect. (D) In contrast, the transgene did not change heart rate ($n = 6$, each, n.s., Tukey's multiple comparisons test following one-way ANOVA), while treatment with L-NA or L-NA/diclofenac resulted in a significant reduction in heart rate ($n = 6$, each, $^{\#}P < 0.05$, Tukey's multiple comparisons test following one-way ANOVA).

Figure 3B the dorsal skin of mice after cutting the skin specimens using a special circular cutter. As shown in Figure 3C, the vast majority of extravasation was found to take place within the first 20 min after injection of bradykinin. In addition, increasing concentrations of bradykinin caused a significant increase in extravasation in 30 min exceeding fourfold

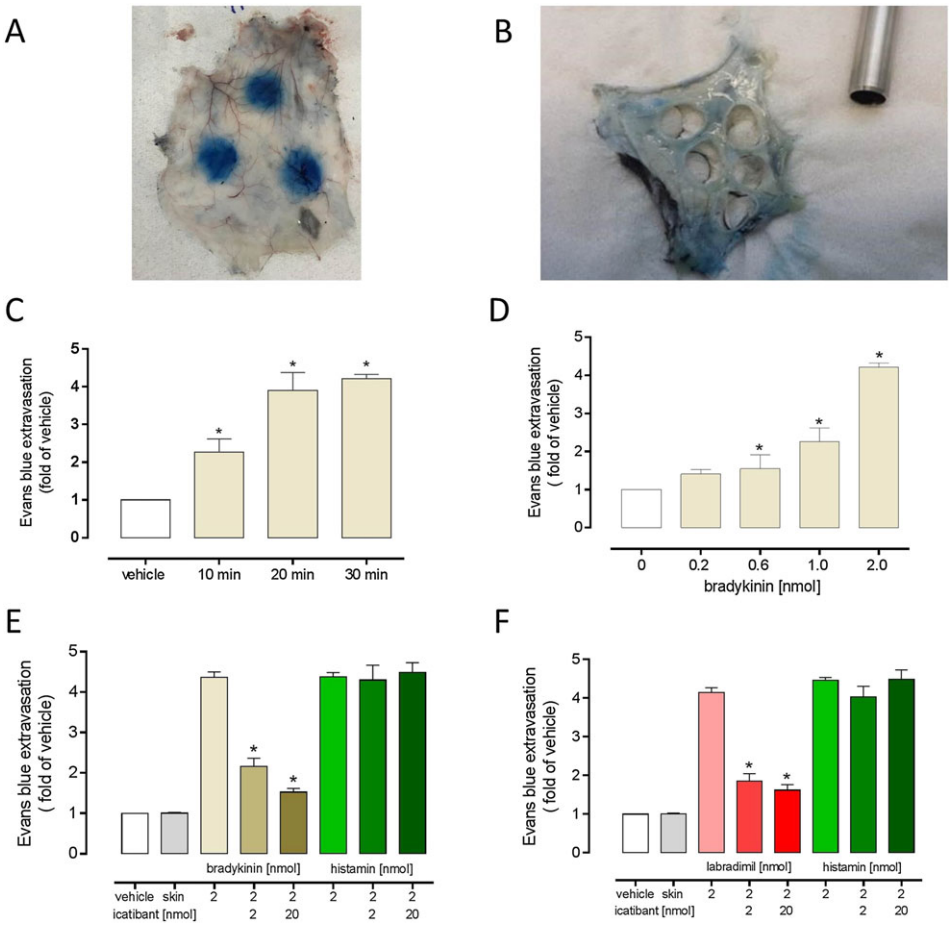

Figure 3

Miles assay to evaluate plasma extravasation induced by i.d. injection of bradykinin in C57BL/6 mice. (A) Illustration of the dorsal skin of mice before and (B) after cutting the skin specimens with a special cutter. (C) Time-dependent extravasation induced by 2 nmol of i.d. bradykinin ($n = 6$ each, $P < 0.05$ one-way ANOVA, $^*P < 0.05$ vs. vehicle, Tukey's multiple comparison test). (D) Concentration-dependent extravasation induced by i.d. bradykinin ($n = 5$ each, $P < 0.05$ one-way ANOVA, $^*P < 0.05$ vs. vehicle, Tukey's multiple comparison test). (E) Inhibition of i.d. bradykinin-induced extravasation by i.v. icatibant in C57BL/6 ($n = 6$ each, $P < 0.05$ one-way ANOVA, $^*P < 0.05$ vs. bradykinin alone, Tukey's multiple comparison test). (F) Inhibition of i.d. labradimil induced extravasation by i.v. icatibant ($n = 6$ each, $P < 0.05$ one-way ANOVA,$^*P < 0.05$ vs. labradimil alone, $^{\#}P < 0.05$ vs. Tukey's multiple comparison test). Icatibant did not block the effect of histamine (vehicle denotes physiological buffer solution, skin denotes for uninjected skin).

of the vehicle value (Figure 3D). A significant increase in extravasation occurred at a dose of bradykinin of 0.6 nmol. Extravasation induced by bradykinin was blocked by icatibant and hence largely mediated by activation of the B_2 receptor (Figure 3E). Similar results were obtained using the specific B_2 agonist labradimil (Figure 3F), which is much more stable against degrading peptidases (Shimuta *et al.*, 1999), and there was no significant difference in the extent of extravasation induced by bradykinin. Of note, icatibant did not block the effect of histamine (Figure 3E, F).

We further investigated whether $B2^{tg}$ shows a greater response to i.d. bradykinin as compared with $B2^{n}$. In $B2^{n}$, we found an increase in extravasation induced by bradykinin of 4.5 ± 0.2-fold ($P < 0.05$ one-way ANOVA, $P < 0.05$ vs. vehicle, Tukey's multiple comparison test), which was comparable with that seen in C57BL/6 mice. In contrast, bradykinin induced a significantly stronger extravasation in $B2^{tg}$ to 5.5 ± 0.2-fold (Figure 4A) suggesting that overexpression of the B_2 receptor in $B2^{tg}$ is functionally active in endothelial cells of small dermal blood vessels. Again, 2-nmol of icatibant almost completely inhibited the extravasation induced by bradykinin as compared with vehicle from 4.5 ± 0.2-fold to 2.3 ± 0.2-fold in $B2^{n}$ ($n = 7$, $P < 0.05$) and from 5.7 ± 0.1-fold to 2.5 ± 0.1-fold ($n = 5$, $P < 0.05$). Furthermore, diclofenac strongly and significantly reduced extravasation in response to bradykinin in both strains (Figure 4B). In addition, diclofenac abolished the difference between $B2^{n}$ and $B2^{tg}$ suggesting a strong contribution of COX products to the augmented extravasation in $B2^{tg}$.

As angio-oedema occurring during treatment with an ACEi is by far the most common cause of non-allergic

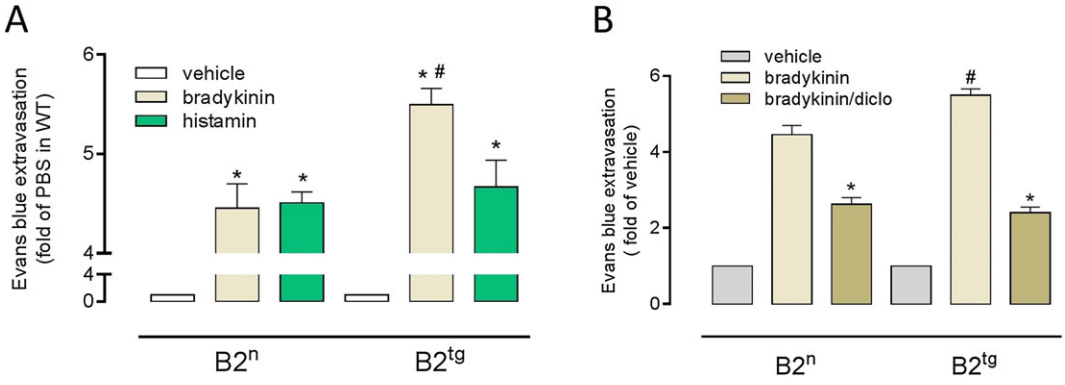

Figure 4

Results from the Miles assay to evaluate plasma extravasation induced by bradykinin in mice harbouring an endothelial-specific overexpression of B_2 receptors ($B2^{tg}$) as compared with their transgene negative littermates ($B2^{n}$). (A) Increased extravasation induced by bradykinin but not histamine in $B2^{tg}$ vs. $B2^{n}$ ($n = 7$ each, vehicle denotes physiological buffer solution, $P < 0.05$ one-way ANOVA,*$P < 0.05$ vs. vehicle, $^{\#}P < 0.05$ vs. $B2^{n}$, Tukey's multiple comparison test). (B) Effect of pretreatment with 5 mg·kg^{-1} diclofenac (diclo) on bradykinin-induced extravasation in $B2^{n}$ and $B2^{tg}$ ($n = 7$ each, $P < 0.05$ one-way ANOVA,*$P < 0.05$ vs. no pretreatment, $^{\#}P < 0.05$ vs. $B2^{n}$, Tukey's multiple comparison test). Note that treatment with diclofenac abolished the difference between $B2^{n}$ and $B2^{tg}$ suggesting little contribution of NO to the augmented extravasation in $B2^{tg}$.

angio-oedema (Bas *et al.*, 2015a), we sought to evaluate whether treatment of C57BL/6 mice with the ACEi moexipril impacts on dose-dependent extravasation induced by i.d. bradykinin. As shown in Figure 5A, moexipril augmented the extravasation induced by an intermediate dose, 0.6 nmol, of bradykinin but had no effect at other doses. This included the lowest dose of bradykinin (0.2 nmol), which produced an increase in extravasation of 1.4 ± 0.1-fold without and 1.4 ± 0.1-fold with moexipril treatment as compared with vehicle. However, in both cases, these small increases did not reach statistical significance as compared with injection of vehicle (n.s. vs. no bradykinin, Sidak multiple comparison test following two-way ANOVA). Another question was whether the significant effect of moexipril on the response to 0.6 nmol of bradykinin might have been caused by vasodilatation induced by this ACEi. To evaluate this, we repeated these experiments using the B_2 agonist labradimil, which is much more stable against degrading peptidases (Shimuta *et al.*, 1999) but has a similar affinity and intrinsic activity as bradykinin. As shown in Figure 5B, moexipril had no effect on extravasation at any concentration of labradimil. These findings suggest that inhibition of ACE by moexipril and not vasodilatation *per se* likely caused the potentiation of extravasation induced by 0.6 nmol of bradykinin.

Two-photon laser microscopy

A 250 kD dextran labelled with a green fluorescent chromophore, which cannot permeate spontaneously, was i.v.

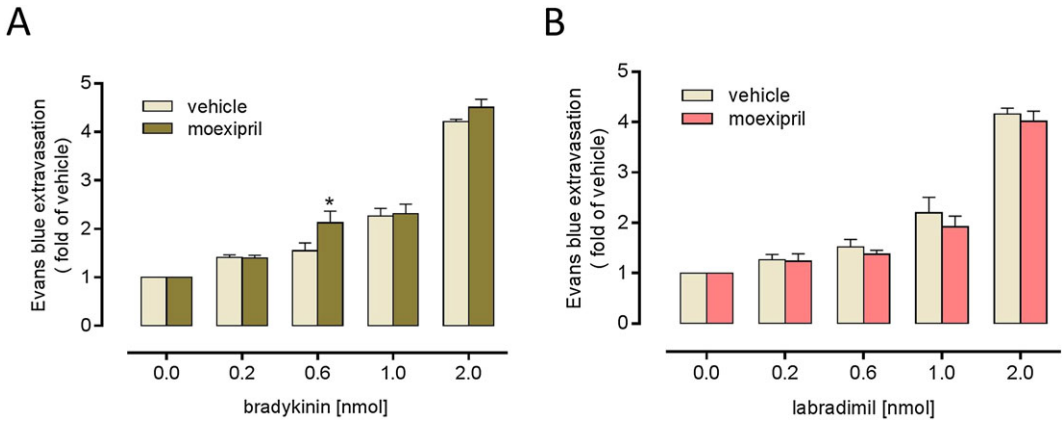

Figure 5

Results from the Miles assay to evaluate plasma extravasation induced by bradykinin in C57BL/6 mice treated with either vehicle or with the ACEi moexipril (0.3 mg·kg^{-1}, i.p.). (A) Increased extravasation induced by bradykinin occurred at an intermediate concentration of bradykinin of 0.6-nmol ($n = 5$ each, vehicle denotes physiological buffer solution, $P < 0.05$ two-way ANOVA, *$P < 0.05$ vs. vehicle, Sidak multiple comparison test). (B) No effect of moexipril on extravasation induced by labradimil occurred at any concentration of labradimil ($n = 5$ each, vehicle denotes physiological buffer solution, $P < 0.05$ two-way ANOVA, n.s. vs. vehicle, Sidak multiple comparison test).

injected. Since the fluorescence stays within the vessel lumen, this probe separates vascular fluorescence from the interstitial signals. After this, a second probe consisting of 10 kD dextran labelled with a red fluorescent chromophore was i.v. injected. This probe left the vascular lumen by permeation through the endothelial barrier during spontaneous extravasation (Figure 6A–E). After 45 min, changes in fluorescence were no longer detectable in the vascular lumen, that is, the signal reached a plateau (Figure 6D). As an additional control, we quantified the accumulation of the 10 kD dextran probe in the interstitial space (Figure 6F). Of note, the accuracy of these measurements is lower, because the interstitial space cannot be defined as clearly as the intravascular space.

Using the methodological approach described above, we investigated whether endothelial overexpression of B_2 receptors would increase spontaneous extravasation as the stimulated extravasation induced by bradykinin was larger in $B2^{tg}$ (Figure 4A). As shown in Figure 7A, there was no difference in the velocity and quantity of extravasation of the 10 kD dextran between C57BL/6 and $B2^{tg}$. Likewise, we could not detect a difference between $B2^{tg}$ and $B2^{n}$ suggesting that spontaneous extravasation in $B2^{tg}$ is not altered. To substantiate these findings, we treated C57BL/6 mice with 2 nmol of icatibant by i.p. injection. As shown in Figure 7C, i.p. administration of icatibant had no effects on spontaneous extravasation. Finally, we investigated whether treatment with moexipril could possibly alter the endothelial barrier and increase spontaneous extravasation as moexipril enhanced the stimulated extravasation induced by 0.6 nmol bradykinin (Figure 5A). As shown in Figure 7D, treatment with 0.3 $mg \cdot kg^{-1}$ moexipril did not change the velocity and quantity of spontaneous extravasation. Taken together, these data suggest that spontaneous extravasation in mice appears to be independent of the activity of B_2 receptors.

Discussion

Overexpression of human B_2 receptors directed to vascular endothelial cells showed several different effects in our mouse model $B2^{tg}$. Despite a more than 15-fold overexpression of human B_2 receptor mRNA, we found no change in murine B_2 receptor mRNA expression in the lungs of $B2^{tg}$ suggesting a strong stability of B_2 receptor expression. In contrast, overexpression of bovine **eNOS** using the same promoter showed a strong reduction of murine eNOS expression (Suvorava *et al.*, 2017). The stability of B_2 receptor expression and function was also observed following acute and prolonged inhibition of endogenous bradykinin formation by a C1 esterase inhibitor or inhibition of B_2 receptors by icatibant (Khosravani *et al.*, 2015). Furthermore, it was shown that prolonged stimulation with inactivation-resistant bradykinin receptor agonists points to a posttranslational mechanism consisting of increased endocytosis of B_2

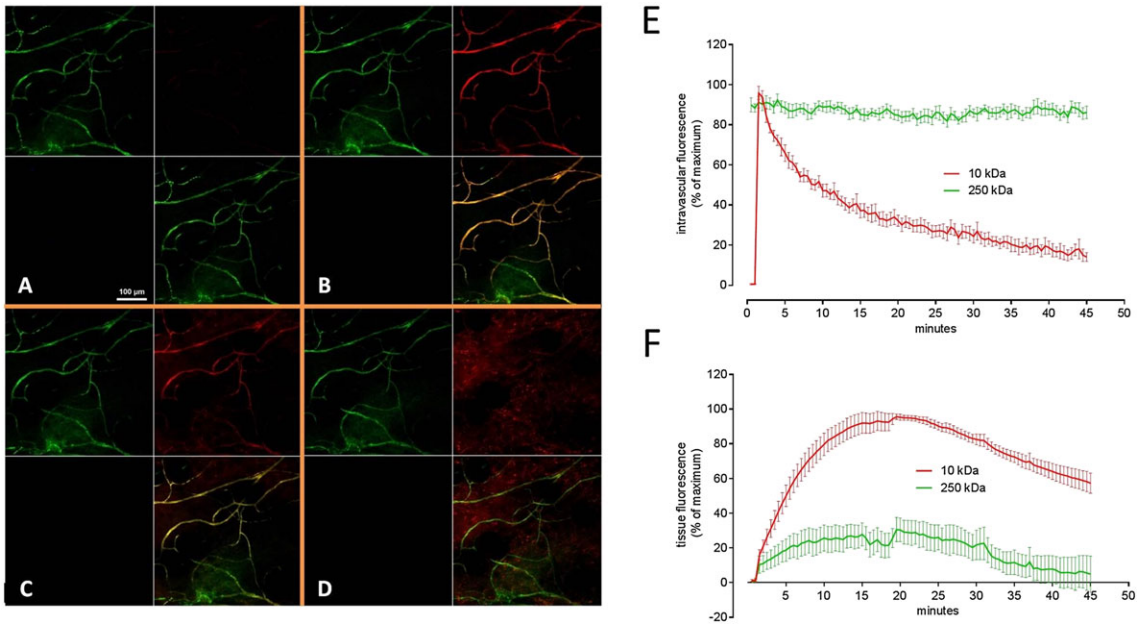

Figure 6

Imaging of small dermal blood vessels of the ear of anaesthetized C57BL/6 mice visualized approximately 200 µm below the surface. Mice were pretreated with 1,5 mg 250 kD dextran labelled with a green fluorescent chromophore. In each of the four pictures, the upper left quadrant shows green fluorescence, the upper right quadrant red fluorescence and the lower right quadrant the overlay in yellow (lower left quadrant is an empty channel). Recordings were taken (A) before injection of 250 µg 10 kD dextran labelled with a red fluorescent chromophore (note the green extravascular auto-fluorescence that does not change over time), (B) before extravasation (time point 0 min, note the red stained vessels and the yellow intravascular overlay), (C) during extravasation (note the red clouds around the vessels) and (D) after 45 min of extravasation (note that the intravascular overlay turned back to green), (E) Quantification of spontaneous extravasation of the 10 kD probe but not the 250 kD probe in C57BL/6 mice ($n = 6$). (F) Quantification of accumulation of the 10 kD probe but not the 250 kD probe in the tissue of C57BL/6 mice ($n = 6$).

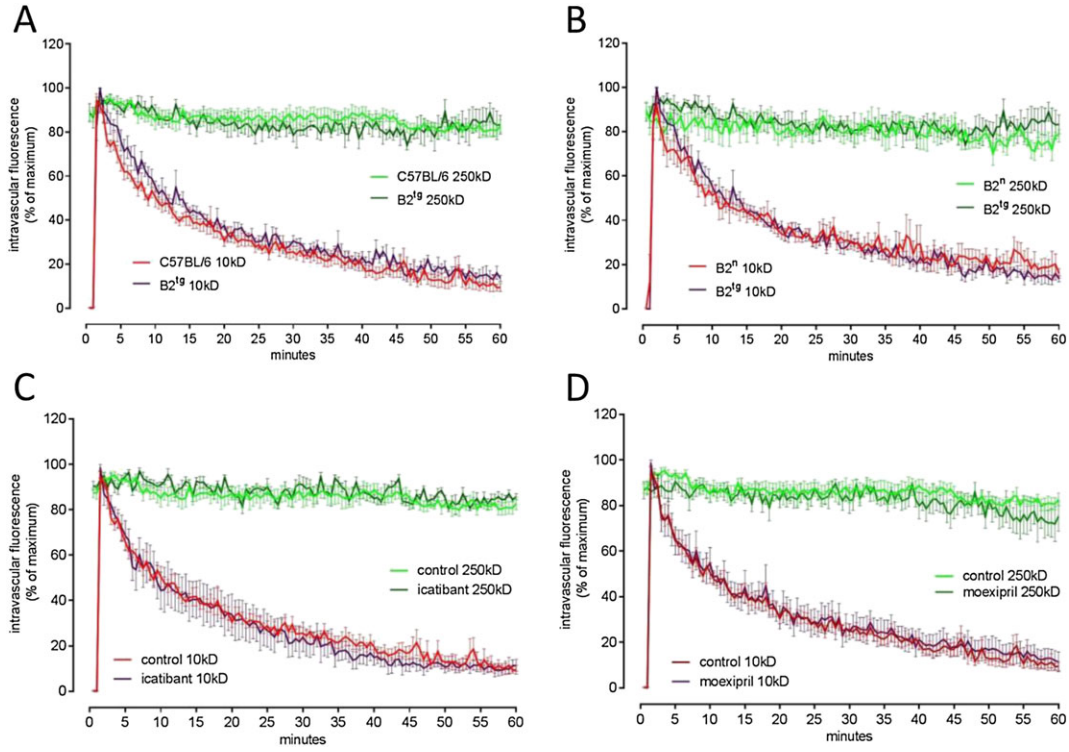

Figure 7

Effect of B_2 receptors on the spontaneous extravasation in C57BL/6 and transgenic $B2^{tg}$ mice. Endothelial-specific overexpression of the B_2 receptor had no effect on the velocity and quantity of extravasation of the red 10 kD dextran probe when compared with (A) C57BL/6 (n = 5, for each time point, Sidak multiple comparison test following two-way ANOVA) or (B) $B2^{n}$ (n = 5, for each time point, Sidak multiple comparison test following two-way ANOVA). (C) Inhibition of the B_2 receptor by icatibant showed no significant difference to control at any time point (Sidak multiple comparison test following two-way ANOVA). (D) I.v. treatment of C57BL/6 with moexipril (0.3 mg·kg^{-1}) did not change the velocity or quantity of spontaneous extravasation (n = 5–7, for each time point, Sidak multiple comparison test following two-way ANOVA).

receptors and their subsequent desensitization and/or degradation (Bawolak *et al.*, 2009).

To characterize our new animal model, we investigated aortic reactivity in response to bradykinin. In $B2^{tg}$, we found a significantly lower vasoconstriction induced by bradykinin suggesting a tonic inhibitory effect of mediated by the overexpression of human B_2 receptors. This might come as a surprise as one would have expected a stronger effect of bradykinin in $B2^{tg}$ aortic rings. However, the vasoconstrictor effect of bradykinin was completely dependent on the activity of COX. Similar findings have been reported to occur in abdominal aortic rings of C57BL/6 mice, which responded to bradykinin with a concentration-dependent constriction that was almost completely abolished by icatibant (Felipe *et al.*, 2007). Furthermore, a similar vasomotor response to B_2 receptor stimulation could be demonstrated in the thoracic aorta of C57BL/6 mice (Khosravani *et al.*, 2015).

We observed as well that inhibition of eNOS by L-NAME potentiated the vasoconstriction induced by bradykinin to a similar extent to the aortic constriction observed in eNOS deficient mice backcrossed to C57BL/6 (Khosravani *et al.*, 2015). These findings suggest that bradykinin activates eNOS and thereby attenuates vasoconstriction by the COX product. However, bradykinin did not dilate aortic rings from C57BL/6 mice (Khosravani *et al.*, 2015) or $B2^{n}$ rings after treatment with diclofenac but this was seen in $B2^{tg}$. This vasodilator response was dependent on intact endothelium, activation of B_2 receptors and activation of eNOS. Hence, it appears that the overexpression of human B_2 receptors in aortic endothelium may have changed the signalling cascade in these cells in favour of the generation of endogenous NO. Interestingly, it has been reported that the B_2 receptor and eNOS are physically associated in the endothelial cell membrane resulting in inhibition of eNOS activity (Ju *et al.*, 1998). Activation of the B_2 receptor induces a rapid dissociation of this heterodimeric protein complex presumably leading to the activation of eNOS. A further study suggested that inhibition of eNOS through the interaction between the B_2 receptor and eNOS blocks the electron transfer of flavin to haem (Golser *et al.*, 2000). Thus, it is conceivable that endothelial-specific overexpression of B_2 receptors may result in a larger number of B_2 receptor-eNOS complexes leading to a sequestration of the total eNOS pool towards B_2 receptors and possibly away from other eNOS inhibiting endothelial proteins such as caveolin (Drab *et al.*, 2001) or the AT_1 receptor (Ju *et al.*, 1998). However, further more detailed molecular studies are needed to substantiate this suggestion.

In a previous investigation, a general overexpression of human B_2 receptors was associated with a hypotensive phenotype suggesting functionally active overexpression

of B_2 receptors in resistance vessels (Wang *et al.*, 1997). A similar drop in blood pressure occurred in this report suggesting that this effect following general overexpression by the Rous sarcoma virus 3′-LTR promoter was mediated by endothelial cells as well. Likewise, B_2 receptor-deficient mice have an increase in systolic and mean blood pressure of about 20 mmHg (Madeddu *et al.*, 1997; Madeddu *et al.*, 1999), which resembles the increase in blood pressure noted following disruption of eNOS (Shesely *et al.*, 1996; Kojda *et al.*, 1999).

As there was no effect of L-NA or diclofenac on the reduction in blood pressure in $B2^{tg}$, we suggest that vasodilatation of resistance blood vessels in $B2^{tg}$ is unlikely to be mediated by activation of eNOS and the subsequent increased generation of NO or the release of vasodilator prostaglandins. Therefore, it seems possible that the reduction in blood pressure in $B2^{tg}$ may result from an increased release of an endothelium-dependent hyperpolarizing factor, which is insensitive to inhibition of eNOS and COXs (Fleming, 2016). For example, bradykinin has been shown to produce 11,12-epoxyeicosatrienoic acid in small porcine coronary arteries (Fisslthaler *et al.*, 1999). Further studies are needed to evaluate the exact mechanisms underlying this aspect of the cardiovascular physiology of $B2^{tg}$.

The pathophysiology of angio-oedema induced by ACEi is mainly mediated by bradykinin-induced activation of vascular B_2 receptors, as treatment with ACEi largely inhibits bradykinin degradation (Bas *et al.*, 2007). The first clinical trial addressing the latter has shown that pharmacological inhibition of ACE increases B_2 receptor activation in hypertensive patients (Gainer *et al.*, 1998). These results strongly suggest that inhibition of ACE dilates resistant vessels by increasing the activation of B_2 receptors. A later study in just a few patients, including one suffering from ACEi-induced angio-oedema, reported that the plasma bradykinin level dropped following cessation of the ACEi (Nussberger *et al.*, 1998). Experimental studies in transgenic mice have consistently shown that the increase in fluid extravasation induced by bradykinin is mediated by the over-activation of B_2 receptors (Han *et al.*, 2002; Bas *et al.*, 2007; Oschatz *et al.*, 2011). Hence, it is not surprising that randomized controlled clinical trials show that the specific B_2 receptor blocker icatibant is effective at reducing the symptom severity and the time to complete resolution of symptoms, that is, the speed of recovery, in hereditary angio-oedema (Cicardi *et al.*, 2010) and in ACEi-induced angio-oedema (Bas *et al.*, 2015b).

We used the Miles assay to quantify dermal extravasation of the albumin-bound dye Evans blue, as initially described by Miles and Miles (1952). This assay has been widely used in the past to study the increase in extravasation in response to i.d. inflammatory stimuli (Nagy *et al.*, 2008). However, the Miles assay has rarely been used to study bradykinin-induced extravasation in mice. In one study, i.d. injection of bradykinin roughly doubled extravasation in C57BL/6 mice within 20 min but no dose-dependency was provided (Benest *et al.*, 2013). Here we showed, by simultaneous injection of increasing concentrations, that bradykinin dose-dependently increases extravasation, and a significant increase was observed at a dose of 0.6 nmol. As expected, increased extravasation to bradykinin was specifically mediated by B_2 receptors. Likewise, injection of labradimil, a B_2 agonist, which is much more stable against degrading peptidases such as ACE (Shimuta *et al.*, 1999), showed almost identical results. Furthermore, we were able to demonstrate that endothelial-specific overexpression of B_2 receptors augmented the extravasation in response to bradykinin suggesting a functional overexpression in small dermal vessels.

We found as well that the unspecific COX inhibitor diclofenac largely decreased the effect of bradykinin on dermal extravasation. Prostaglandins such as PGE_2 are known to potentiate the effects of bradykinin on endothelial permeability while inducing very little extravasation if injected without bradykinin. For example, concomitant i.d. injection of prostaglandins and bradykinin in rabbits produces a much larger fluid extravasation, that is, oedema formation, than bradykinin alone (Williams and Morley, 1973). This has been attributed to the vasodilator activity of PGE_2 that increases blood flow in the region of injection (Williams and Peck, 1977). A more recent study demonstrated a strong inhibition of dermal extravasation induced by the combination of bradykinin/PGI_2 in PGI_2-receptor (IP receptor) deficient mice as compared with transgene negative mice, but a possible influence of IP receptor deficiency on the response to bradykinin alone was not investigated (Murata *et al.*, 1997). Another study in FVB/NJcl mouse ear skin reported that injection of PGE_2 induced increased extravasation that was mediated by activation of the E-prostaglandin receptors EP_2 and EP_4 (Omori *et al.*, 2014). Although these thorough studies clearly revealed the important role of prostaglandins in plasma extravasation under inflammatory conditions, no investigation had been undertaken so far to evaluate the role of prostaglandins generated in response to bradykinin during bradykinin-induced oedema formation. However, our data strongly suggest that prostaglandin formation in response to activation of B_2 receptors in small dermal blood vessels of mice might contribute to the increase in vascular permeability.

We wished to obtain experimental evidence for the hypothesis that pharmacological inhibition of ACE may decrease the endothelial barrier function by increasing the steady-state plasma levels of bradykinin using a functional approach. We found that moexipril augmented extravasation induced by an intermediate dose of bradykinin but not that induced by higher or lower doses. It might be argued that high i.d. concentrations of bradykinin overshadow the effect of ACE on bradykinin activity. However, it remains to be elucidated why moexipril had no effect at the lowest dose of bradykinin. We speculated that this might be a consequence of a too low dose of moexipril. However, at higher doses moexipril induced a reduction in blood pressure resulting in decreased extravasation induced by bradykinin and by histamine, presumably because of centralization of blood flow. It has been reported that a dose of 2,5 $mg \cdot kg^{-1}$ captopril reduces the mean blood pressure in mice by about 40 mmHg to less than 80 mmHg (Emanueli *et al.*, 1998). At a dose of moexipril corresponding to this dose of captopril neither bradykinin nor histamine induced any detectable extravasation, not even at the highest i.d. dose of 2 nmol per injection. To this end, our data suggest that inhibition of ACE augments extravasation induced by bradykinin.

While the Miles assay appears to be a reliable method to measure changes in dermal extravasation provoked by different interventions, it has certain limitations (Nagy *et al.*, 2008). For example, the assay is not suitable for quantifying spontaneous extravasation. In contrast, the two-photon laser methodology offers this advantage and allows the spontaneous extravasation of the red fluorescent 10 kDa probe from the intravascular space into the interstitial space to be studied in a continuous manner, as reported previously (Egawa *et al.*, 2013). We used three different approaches to evaluate a possible contribution of B_2 receptors to spontaneous extravasation, that is, the effect of functionally active overexpression of the receptor in small dermal skin vessels, the effect of icatibant and the effect of moexipril. No changes in the kinetics of spontaneous extravasation were observed in any of these settings. However, the accuracy of measurements on accumulation of both probes in the tissue is less reliable as compared with the intravascular space. This is the result of a less accurate focus as it is impossible to set predefined measurement areas. In the intravascular space, this is done after injection of the 250 kDa green fluorescent dye, which exactly indicates the area of measurement. In striking contrast, measurements within the tissue itself are more diffuse. This includes measurement of extravascular auto-fluorescence shown in Figure 6A–D, which does not change over time and cannot be subtracted correctly. For this reason, tissue data are not shown in Figure 7. Taken together, these data suggest that activation of B_2 receptors is unlikely to contribute to spontaneous extravasation in mice.

Our study has some limitations. Firstly, the significance of our data for humans is not known. Secondly, we cannot differentiate between the known extravasation routes. In general, plasma fluid and proteins can traverse the endothelial barrier by a para-cellular and a transcellular route, and both routes are involved in physiological and pathophysiological extravasation, that is, the development of oedema. Para-cellular extravasation in capillaries, which mainly transports fluid and small molecules, occurs through inter-endothelial junctions (continuous capillaries) and/or endothelial cell pores (fenestrated capillaries) (Mehta and Malik, 2006), and both types of capillaries are present in human skin (Takada and Hattori, 1972). In contrast, the transcellular route (transcytosis) accounts for the majority of protein transport across the endothelial barrier, for example, the most abundant interstitial protein albumin. This route is characterized by vesicles that were discovered and linked to extravasation by George E. Palade (Majno and Palade, 1961; Weibel and Palade, 1964). Finally, we currently do not know whether mediators other than prostaglandins and/or changes in the endothelial glycocalyx contribute to the extravasation induced by bradykinin. Further studies are warranted to investigate this in more detail.

Taken together, the results of our study suggest that activation of B_2 receptors by endogenous bradykinin is unlikely to contribute to physiological extravasation. Therefore, the assumption that treatment with an ACEi results in an alteration in the physiological vascular barrier function predisposing to non-allergic angio-oedema is not supported by our findings.

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Author contributions

M.Bi. did Miles assays and two-photon laser studies and contributed to collection and assembly of data and writing of the manuscript, V.T.-V.D. cloned the B_2 receptor plasmid and established the genotyping PCR. E.G.F. and F.K. contributed to the Miles assays and two-photon laser experiments. M.V. and Mv.Z. helped to establish the two-photon laser technique, S.W. did the quantitative real-time PCR. M.Ba. contributed to the cloning of the B_2 receptor plasmid and provided financial support. T.S. performed the organ bath experiments and the blood pressure measurements, data analysis and interpretation and contributed to the writing of the manuscript. G.K. conceived and designed the study, provided administrative support, data analysis and interpretation, and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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