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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Stability of murine bradykinin type 2 receptor despite treatment with NO, bradykinin, icatibant, or C1-INH

F. Khosravani^{1,*}, T. Suvorava^{1,*}, V. T.-V. Dao¹, N. Brockmann², O. Kocgirli¹, F. F. Herbst¹, S. Valcaccia¹, M. U. Kassack², M. Bas³ & G. Kojda¹

¹Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine-University; ²Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University, Düsseldorf; ³Otorhinolaryngology Department, University Hospital Rechts der Isar, Munich Technical University, Munich, Germany

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Keywords

bradykinin type 2 receptor; C1-INH; endothelial nitric oxide synthase; icatibant; nitric oxide donor.

Correspondence

Georg Kojda, PharmD, PhD, Institut für Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universität, Moorenstr. 5, 40225 Düsseldorf, Germany.
Tel.: +49-211-81-12518
Fax: +49-211-81-14781
E-mail: kojda@uni-duesseldorf.de

*Both authors contributed equally to this work

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Abstract

Background: Little is known about factors which trigger and/or contribute to hereditary angioedema or ACE-inhibitor-mediated angioedema including variations in bradykinin type 2 receptor (B2R) expression and activity.

Methods: Protein and mRNA expression of B2R and the increase of intracellular calcium (iCa) in response to bradykinin were monitored in porcine and murine endothelial cells in response to NO donors or bradykinin. B2R protein expression was evaluated in skin, heart, and lung of (i) mice with endothelial-specific overexpression of eNOS (eNOS^{tg}), (ii) in eNOS^{-/-} mice and (iii) in C57BL/6 mice treated with the NO donor pentaerythritol tetranitrate (PETN), the NOS inhibitor L-nitroarginine (L-NA), plasma pool C1-INH, and the B2R antagonist icatibant. Aortic reactivity to bradykinin was investigated including eNOS^{-/-} mice.

Results: B2R protein and mRNA expression remained unchanged in cells subjected to L-NA, NO donors, and bradykinin in a time- and concentration-dependent manner. Likewise, increases of iCa in murine brain endothelial cells remained unchanged. B2R protein levels were similar in eNOS^{tg} and eNOS^{-/-} as compared to transgene-negative littermates. Likewise, treatment of C57BL/6 mice with PETN, L-NA, C1-INH or icatibant did not change B2R protein expression. In aortic rings of C57BL/6 mice, bradykinin induced B2R-dependent constrictions which were attenuated by endothelial NO and abolished by diclofenac indicating the functional importance of B2R-induced activation of endothelial NO synthase and cyclooxygenase.

Conclusion: These data suggest that alterations of B2R protein expression induced by NO, bradykinin, C1-INH, or icatibant unlikely contribute to bradykinin-induced angioedema. This finding does not rule out a role for NO in bradykinin-induced extravasation and/or angioedema.

Angioedema is a swelling of the mucosa and/or submucosa of the skin which may impair breathing and is potentially life threatening (1). Swellings of the head and neck region, particularly in the pharynx and the larynx, often require emergency treatment and several days of hospitalization. Nonallergic angioedema might be caused by hereditary disposition or is of iatrogenic origin. According to published reports, the vast majority of these forms of angioedema are induced by increased bradykinin levels (2). This might be due to overproduction of bradykinin as in hereditary angioedema (HAE) and acquired angioedema. Another important cause is

decreased degradation of bradykinin by inhibition of certain proteases and receptors of the renin-angiotensin-aldosterone system termed (RAAS)-blocker-induced angioedema (RAE). This is known to occur following pharmacotherapy with ACE inhibitors (ACEi) and possibly angiotensin type 1 receptor blockers (1). In view of the reported frequencies of ACEi use and the occurrence of angioedema as a side-effect (0.1–0.4%), angioedema induced by ACEi is much more common than HAE.

The role of increased formation of bradykinin in the pathophysiology of HAE- and ACEi-induced angioedema is now

well accepted. However, this understanding does not explain the often poor correlation between C1 esterase inhibitor (C1-INH) activity in plasma and the frequency of attacks (3). It does also not explain that only about 0.5% of patients receiving ACEi report an episode of angioedema. Likewise, HAE patients do not report dry cough, while this is a frequent side-effect of ACEi treatment ascribed to bradykinin accumulation in the bronchi (4). Moreover, in very few cases, long-term pharmacologic therapy increased the attack frequency in several HAE patients (5).

The role of alterations of bradykinin activity in HAE and RAE is less well understood. This includes the regulation of the expression of B2R. Likewise, little is known about changes of B2R sensitivity following prolonged stimulation with bradykinin. The same holds true for vascular NO generated in response to activation of B2R (1). Furthermore,

treatment with ACEi increases endothelium-dependent vasodilation induced by acetylcholine and/or hyperemia indicating increased vascular NO bioavailability in cardiovascular patients. This has been shown in hypertension (6), non-insulin-dependent diabetes (7), and coronary artery disease (8). The regulation of B2R might contribute to HAE and RAE as well as to the efficacy of drugs such as C1-INH or icatibant. In addition, such regulation might contribute to dry cough induced by ACEi (4).

The aim of this study was to evaluate the role of NO, bradykinin, C1-INH, or icatibant for the regulation of B2R gene expression and activity. The regulation of expression and activity of vascular B2R cannot be studied directly in humans. Thus, our experimental setup consisting of investigations in endothelial cells of different species and several organs of mice including lung, thoracic aorta, and skin tissue

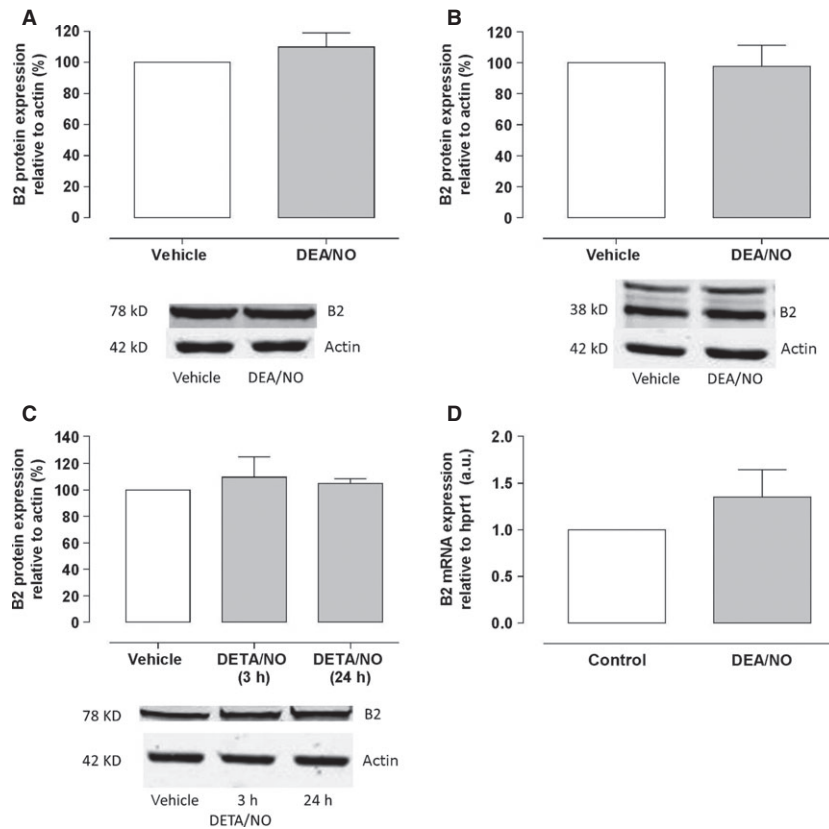


Figure 1 Effects of NO on B2R protein expression in endothelial cells: (A) B2R protein expression as assessed with the Abcam antibody in bEND.3 incubated with 10 μ M DEA/NO for 3 h ($109.9 \pm 9.1\%$, $n = 8$) as compared to controls (100%, $n = 8$, $P = 0.3028$) or (B) assessed with the BD antibody ($97.61 \pm 13.63\%$, $n = 8$) as compared to vehicle (100%, $n = 8$, $P = 0.8630$) (C) B2R protein expression as assessed with the Abcam antibody in bEND.3 incubated with 1 mM DETA/NO (a NO donor leading to a sustained release of NO over 24 h), for 3 h ($109.6 \pm 15.42\%$, $n = 8$) and for 24 h ($104.8 \pm 3.67\%$, $n = 6$) as compared to vehicle ($P = 0.8014$ One-way ANOVA) (D)

B2R mRNA expression as assessed by RT-PCR using primers specific for murine B2R and hprt1 in bEND.3 incubated with 10 μ M DEA/NO for 3 h (1.35 ± 0.29 a.u.) (1.0 a.u., $n = 5$, $P = 0.2245$). All comparisons are based on the analysis of signals on the same blot. All data are means $\pm$ SEMs, and indicated P -values are calculated by unpaired two-tailed Student's t -test or by one-way ANOVA (a.u. = arbitrary units for fluorescence intensity). Representative Western blot examples are depicted at the bottom of the corresponding graphs (blots were cut to control for protein load). Examples of full blots are shown in Supporting Information.

was chosen as a model to show variations in both expression and activity of B2R including *in vivo* approaches.

Materials and Methods

Mice

Mice deficient for endothelial nitric oxide synthase (eNOS^{-/-}) (9) and mice with endothelial-specific overexpression of eNOS (eNOS^{tg}) were used in this study (10,11). Transgene-negative littermates (eNOSⁿ) served as controls. In addition, C57BL/6 mice were used as nontransgenic controls. C57BL/6 mice received placebo, PETN, or N^ω-nitro-L-arginine. All mice were males and 12–20 weeks of age. Permission for the animal studies was provided by the regional government of Germany (AZ 50.05-230-3-65/99, AZ 50.05-230-3-94/00, AZ 50.05-230-18/06). The experiments were performed according to the guidelines for the use of experimental animals, as given by the German 'Tierschutzgesetz' and the 'Guide for the Care and Use of Laboratory Animals' of the US National Institutes of Health.

Cell culture and incubation experiments

Porcine aortic endothelial cells (PAEC) were isolated from fresh porcine thoracic aortae. Murine brain endothelial cells (bEnd.3) were purchased. For all experiments, PAEC of passage 2–3 and bEND of passages 24–32 were used. Standard media were replaced by media without fetal bovine serum, and cells were subjected to the NO donors DEA/NO for 3 h and DETA/NO (100 μ M) for 24 h.

Western blotting

Western blot analysis was carried out in a blinded fashion as described previously (12) using commercially available mouse monoclonal antibodies directed against B2R or eNOS and a rabbit monoclonal anti-B2R antibody (Abcam, Cambridge, UK). Total protein levels were determined by the Bradford method.

Real-time reverse transcription–polymerase chain reaction

Total RNA from bEND.3 was isolated using the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany) and real-time RT-PCR was performed with TaqMan[®] Gene Expression Assays (Applied Biosystems, Weiterstadt, Germany) using Mm01339967_m1 and Mm00446968 for mouse B2R and hprt1. B2R mRNA expression levels relative to hprt1 were determined using the $\Delta\Delta C_t$ method and expressed in arbitrary units.

Ca²⁺ measurements in bEnd.3 cells

Cells (20 000–35 000 cells/well) were loaded with a membrane permeable fluorescent dye (Oregon Green 488 BAPTA-1AM; Molecular Probes, Eugene, OR, USA), and Ca²⁺ fluorescence measurements were performed using a NOVOstar[®] plate

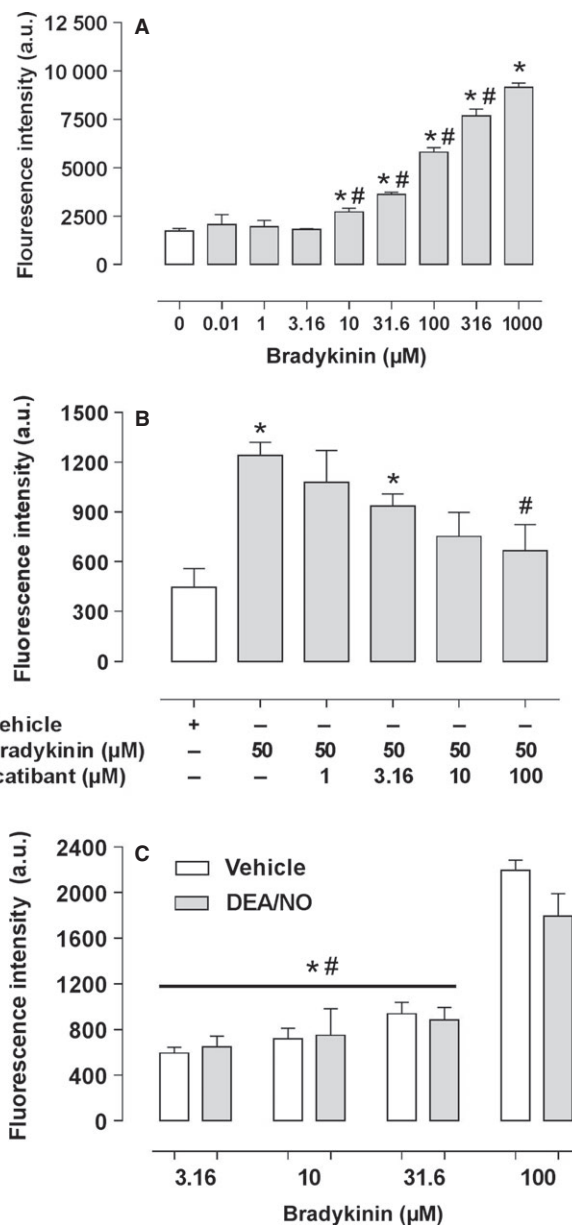


Figure 2 (A) Bradykinin increased iCa²⁺ in bEnd.3 cells in a concentration-dependent manner ($P < 0.0001$ one-way ANOVA, $*P < 0.05$ vs control, $#P < 0.05$ next concentration steps). ATP (10 μ M) was used as a positive control and induced a signal of 9643 ± 153 a.u. (B) The increase of iCa²⁺ induced by 50 μ M bradykinin was inhibited by the B2R antagonist icatibant in a concentration-dependent manner indicating dependency of B2R ($P = 0.0066$ one-way ANOVA, $*P < 0.05$ vs vehicle, $#P < 0.05$ vs 50 μ M bradykinin). (C) The increase of iCa²⁺ in response to bradykinin showed no difference after preincubation for 3 h with the NO donor DEA/NO (10 μ M) in bEnd.3 cells ($P < 0.0001$ one-way ANOVA, $*P < 0.05$ vs 100 μ M bradykinin, $#P < 0.05$ vs 100 μ M bradykinin after 3 h DEA/NO). All data are means $\pm$ SEMs, and indicated P -values are calculated by Newman–Keuls test following one-way ANOVA (a.u. = arbitrary units for fluorescence intensity).

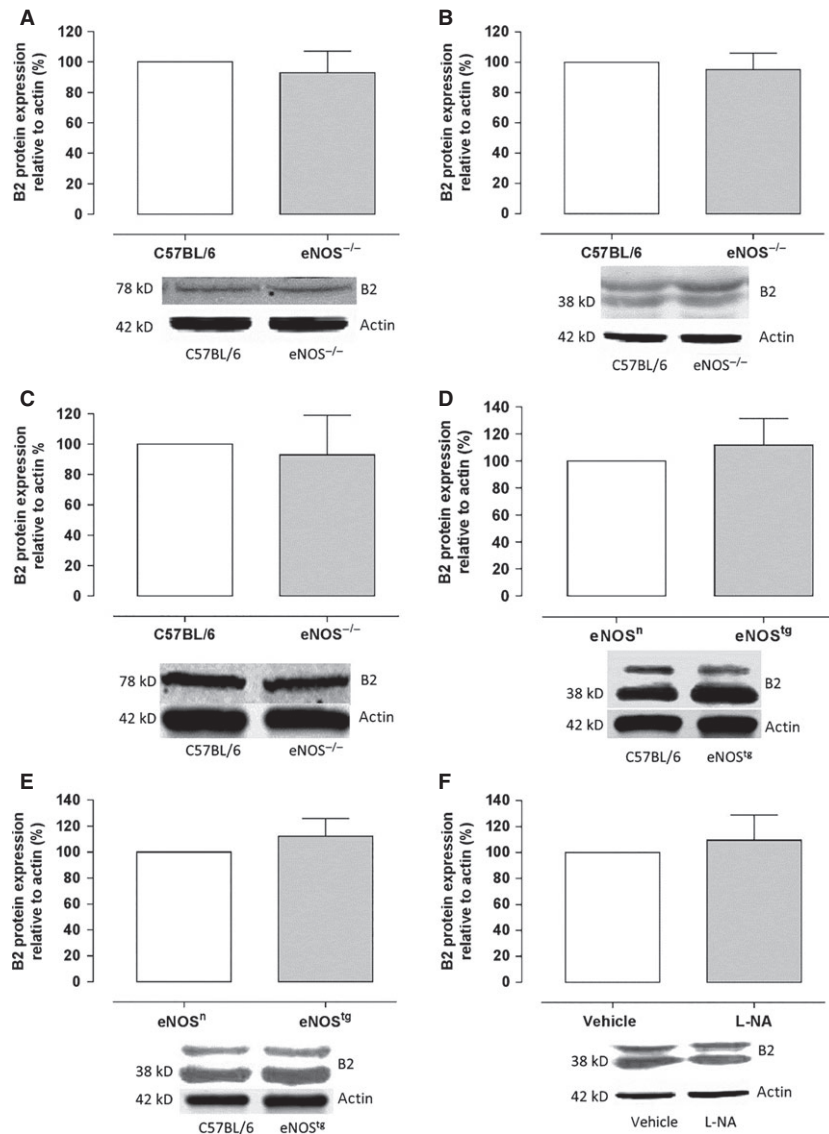


Figure 3 B2R protein expression in skin, lung, and left ventricular myocardial tissue of mice. B2R protein expression in dorsal skin tissue of eNOS^{-/-} mice (A) evaluated with the Abcam antibody ($97.61 \pm 13.63\%$, $n = 4$) as compared to C57BL/6 mice (100%, $n = 4$, $P = 0.6380$) and (B) evaluated with the BD antibody ($95.25 \pm 10.87\%$, $n = 4$) as compared to C57BL/6 mice (100%, $n = 4$, $P = 0.6775$). B2R protein expression in lung tissue of (C) eNOS^{-/-} mice evaluated with the Abcam antibody ($93.0 \pm 26.0\%$, $n = 5$) as compared to C57BL/6 mice (100%, $n = 5$, $P = 0.7930$) and of (D) eNOS^{tg} mice evaluated with the BD antibody ($111.6 \pm 18.4\%$, $n = 12$) as compared to the transgene-negative littermates eNOSⁿ (100%, $n = 12$, $P = 0.5639$). B2R protein expression in left ventricular myocardial tissue of (E) eNOS^{tg} mice

($112.2 \pm 13.7\%$, $n = 10$) as compared to eNOSⁿ (100%, $n = 10$, $P = 0.3968$) and of (F) C57BL/6 mice treated with the NOS inhibitor L-NA ($111.6 \pm 18.4\%$, $n = 12$) as compared to vehicle-treated controls (100%, $n = 12$, $P = 0.5639$) both evaluated with the BD antibody. The strain eNOS^{tg} is characterized by a 3.3-fold endothelial-specific overexpression of eNOS (10). All comparisons are based on the analysis of signals on the same blot. All data are means $\pm$ SEMs, and indicated P -values are calculated by unpaired two-tailed Student's t -test. Representative Western blot examples are depicted at the bottom of the corresponding graphs (blots were cut at about 55 kD and stained with the different antibodies, and the lower part was stripped to control for protein load). Examples of full blots are shown in Supporting Information.

reader (BMG Lab Technologies, Offenburg, Germany). Concentration response curves were performed by adding 10 nM–1 mM bradykinin following the treatment with vehicle, icatibant (30 min), or DEA/NO (3 h). Addition of ATP (10 μ M) served as initial control for the cellular Ca^{2+} response.

Aortic reactivity studies

Function of the endothelium was examined by cumulative addition of acetylcholine (ACh, 0.01–10 μ M) as described previously (10). In some experiments, the endothelium was

removed by gently rubbing the intimal surface with a small wooden stick. Bradykinin (0.01–10 μ M) was added by cumulative application after precontraction with phenylephrine (0.2 μ M).

Statistics

All data were analyzed by standard computer programs (GRAPHPAD PRISM PC Software, Version 3.0, GraphPad Software Inc., La Jolla, CA, USA, analysis of variance, ANOVA) and a *P*-value below 0.05 was considered as significant.

A more detailed description of Materials and Methods is available in the Supporting Information.

Results

Effect of NO on endothelial B2R protein expression in cultured cells

Western blot analysis using two different B2R antibodies revealed no effect of the NO donor DEA/NO on B2R protein expression in bEND.3 (Fig. 1A) and PAEC (Fig. 1B). Similar results were obtained with the NO donor DETA/NO (Fig. 1C). Likewise, DEA/NO did not change mRNA level as evaluated by RT-PCR (Fig. 1D). Finally, treatment of PAEC with 100 μ M bradykinin only slightly increased B2R expression to $122.8 \pm 21.7\%$ ($n = 6$, control set to 100%, $P > 0.05$). These data suggest that neither NO nor bradykinin alter the expression of B2R in endothelial cells. Both antibodies were evaluated for B2R and for species specificity. The results are shown in and described in the Figs S1 and S2.

Effect of NO on B2R-induced Ca^{2+} signaling

As shown in Fig. 2A, increasing concentrations of bradykinin induced increasing concentrations of iCa^{2+} . The maximal response obtained with bradykinin was similar to the response observed with ATP which was used as a positive control. The experiments were repeated using a constant concentration of 50 μ M of bradykinin following treatment with increasing concentrations of icatibant. This resulted in strongly reduced increases of iCa^{2+} demonstrating B2R dependency (Fig. 2B). To investigate whether NO significantly influenced the activity of B2R, bEnd.3 cells were pretreated with DEA/NO. Subsequent stimulation with increasing concentrations of bradykinin demonstrated that NO does not change iCa^{2+} in response to bradykinin (Fig. 2C), suggesting that NO has no effect on phospholipase C signaling induced by B2R.

Effect of vascular NO bioavailability on B2R protein expression *in vivo*

B2R expression was assessed in $\text{eNOS}^{-/-}$ mice, and a transgenic mouse strain was characterized by 3.3-fold endothelial-specific overexpression of eNOS (10). B2R protein levels were similar in dorsal skin tissue of $\text{eNOS}^{-/-}$ and C57BL/6 mice as evaluated with both antibodies (Fig. 3A,B). Likewise, B2R

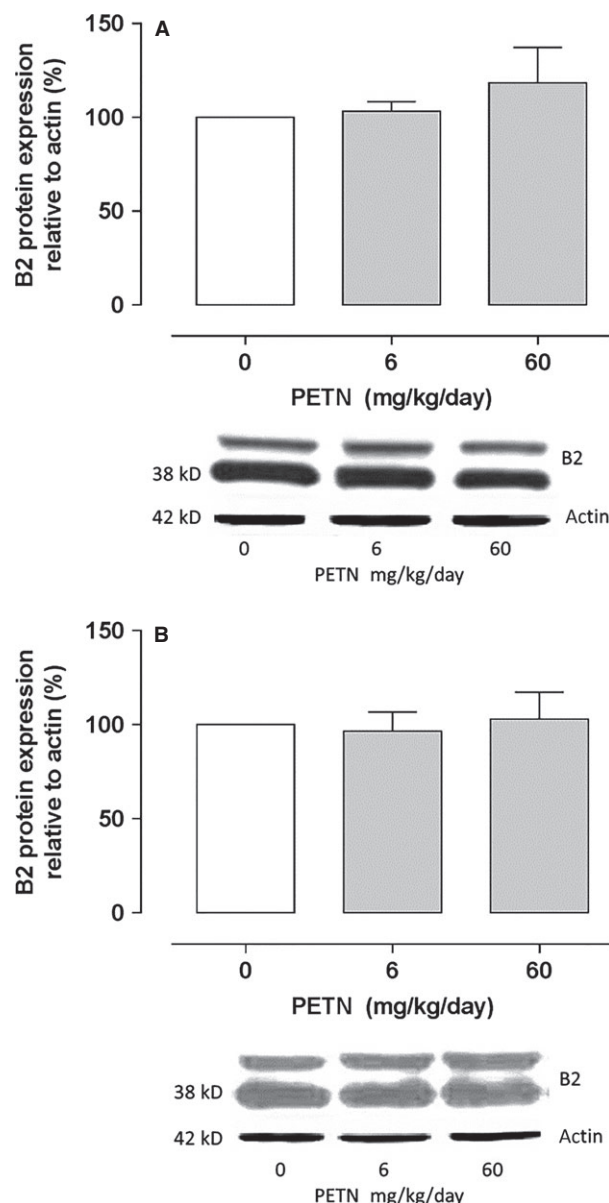


Figure 4 B2R protein expression in lung and left ventricular myocardial tissue of mice treated with the NO donor pentaerythritol tetranitrate (PETN). B2R protein expression evaluated with the BD antibody in (A) lung tissue of C57BL/6 mice treated for 4 weeks with 6 mg/kg/day ($103.2 \pm 5.28\%$, $n = 5$) and 60 mg/kg/day ($118.4 \pm 18.9\%$, $n = 5$) as compared to vehicle (100%, $n = 5$, $P = 0.3916$) and (B) in left ventricular myocardial tissue of C57BL/6 mice after treatment for 4 weeks with 6 mg/kg/day ($98.6 \pm 10.1\%$, $n = 6$) and 60 mg/kg/day ($103.0 \pm 14.3\%$, $n = 6$) as compared to vehicle (100%, $n = 6$, $P = 0.8602$). Treatment with PETN showed no difference to treatment with vehicle. All comparisons are based on the analysis of signals on the same blot. All data are means $\pm$ SEMs, and indicated *P*-values are calculated by one-way ANOVA. Representative Western blot examples are depicted at the bottom of the corresponding graphs (blots were stripped to control for protein load). Examples of full blots are shown in Supporting Information.

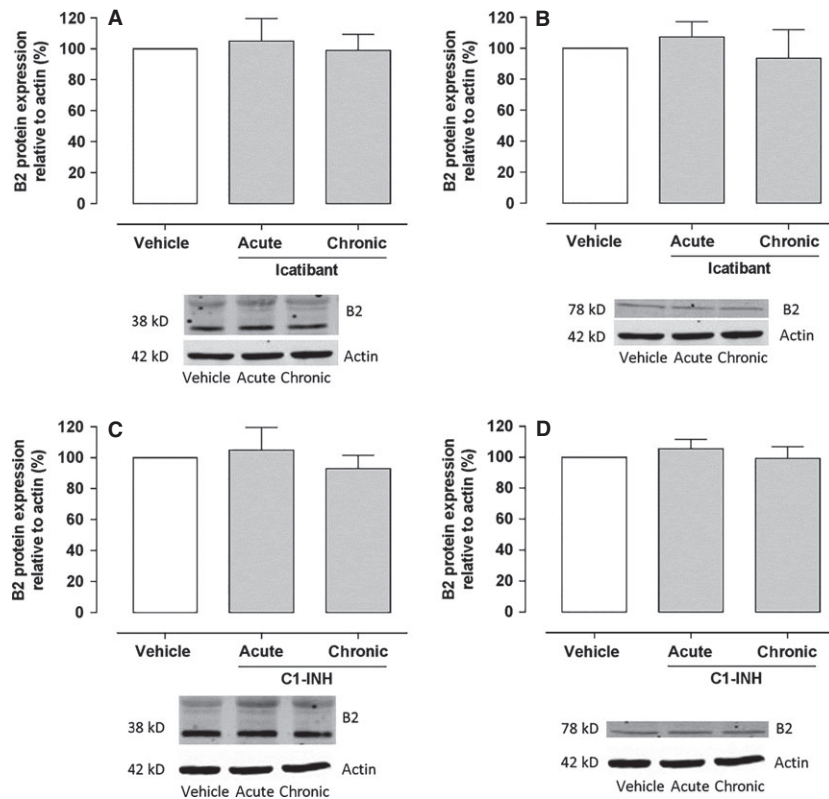


Figure 5 B2R protein expression in lung tissue of C57BL/6 mice 3 h after a single injection (acute) and 3 h after the last of three i.v. injections on three consecutive days (chronic) with the B2R inhibitor icatibant or the kallikrein inhibitor C1-INH ($n = 5$ each). (A) B2R protein expression following treatment with icatibant as detected with the BD antibody ($P = 0.1010$) or (B) the Abcam antibody ($P = 0.3298$). (C) B2R protein expression following treatment with C1 esterase inhibitor (C1-INH) as detected with the BD antibody

($P = 0.3855$) or (D) the Abcam antibody ($P = 0.3884$). All comparisons are based on the analysis of signals on the same blot. All data are means $\pm$ SEMs, and indicated P -values are calculated by one-way ANOVA. Representative Western blot examples are depicted at the bottom of the corresponding graphs (blots were cut at about 55 kD and stained with the different antibodies, and the lower part was stripped to control for protein load). Examples of full blots are shown in Supporting Information.

protein levels were not different in lung tissue of $eNOS^{-/-}$, $eNOS^{tg}$ (Fig. 3C,D) and in left ventricular myocardium of $eNOS^{tg}$ (Fig. 3E) as compared with C57BL/6 mice. Similar results were obtained in ventricular myocardium following 4 weeks treatment of C57BL/6 mice with L-NA (Fig. 3F) or with the NO donor and organic nitrate PETN at two different doses (Fig. 4) (11). Taken together, these data confirm the *in vitro* data and suggest that B2R expression is not regulated by changes of vascular NO bioavailability in mice.

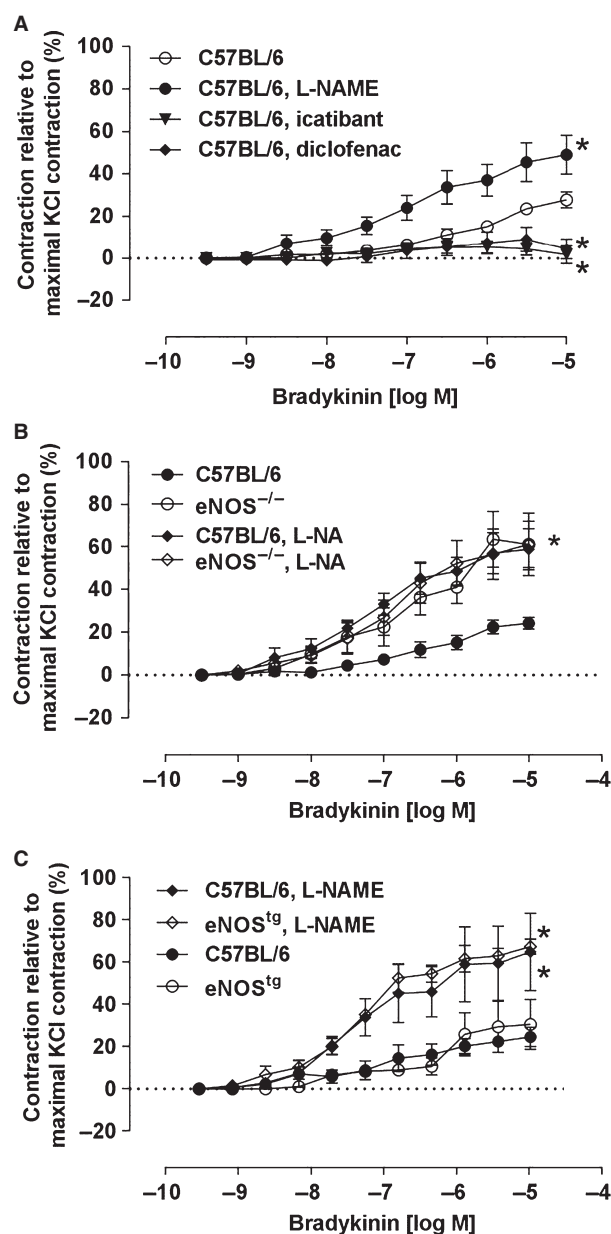
Effect of endogenous vascular bradykinin on B2R protein expression *in vivo*

To study the effect of bradykinin on vascular B2R protein expression *in vivo*, six groups of C57BL/6 mice were treated once (acute) and twice (chronic) with either icatibant competitively inhibiting binding of bradykinin to B2R or C1-INH which inhibits kallikrein and thereby the generation of endogenous bradykinin. Neither icatibant nor C1-INH

showed a significant effect (Fig. 5), suggesting that drug-induced variations in endogenous B2R activity do not alter B2R expression in mice.

Vascular B2R activity

Bradykinin induced concentration-dependent aortic constrictions with a maximal effect at 1 μ M of bradykinin (Fig. 6A). Removal of the endothelium strongly reduced the maximal constriction from $25.2 \pm 2.4\%$ of the maximal KCl response to $7.9 \pm 2.1\%$, while treatment with L-NA increased the contractile response (Fig. 6B). The contraction due to bradykinin was completely inhibited by icatibant or diclofenac (Fig. 6A), indicating that it is mediated by endothelial B2R activation and dependent on cyclooxygenase activity. In $eNOS^{-/-}$, aortic constrictions to bradykinin were similar to that obtained in aortic rings treated with L-NA. Finally, aortic vasoreactivity to B2R activation was not different between C57BL/6 mice and $eNOS^{tg}$, although the latter expresses much more eNOS in endothelial cells (Fig. 6C). These data



suggest that changes of vascular NO bioavailability in mice can alter B2R activity without changing its expression.

Discussion

The regulation of B2R gene expression and activity is not well understood. However, such a regulation may contribute to overstimulation of B2R which is known to mediate to HAE and RAE (1). The aim of this study was to investigate whether alterations of B2R activity and the steady-state levels of NO may change B2R expression. To address this question, we investigated endothelial cells treated with NO donors and bradykinin, mice with genetic variations in vascular NO

Figure 6 Activity of bradykinin in aortic rings of mice. (A) Bradykinin induced a constriction in aortic rings of C57BL/6 mice ($n = 9$) which was dependent on the concentration ($P < 0.0001$, two-way ANOVA) and on inhibition of eNOS by 100 μ M L-NAME ($n = 10$), inhibition of cyclooxygenase by 10 μ M diclofenac ($n = 4$), or blockade of B2R by 10 μ M icatibant ($n = 4$) added to the organ bath ($P < 0.0001$, two-way ANOVA, $*P < 0.05$ vs C57BL/6 mice, Newman-Keuls test). (B) Bradykinin induced a constriction in aortic rings of C57BL/6 mice ($n = 4$) and eNOS^{-/-} ($n = 4$) which was dependent on the concentration ($P < 0.0001$, two-way ANOVA) and on the mouse strain ($P < 0.0001$, two-way ANOVA, $*P < 0.05$ vs C57BL/6 mice, Newman-Keuls test). Treatment of aortic rings with L-NAME augmented the constriction in C57BL/6 mice ($*P < 0.05$ vs C57BL/6 mice, Newman-Keuls test, $n = 4$) but not in eNOS^{-/-} ($P > 0.05$, Newman-Keuls test, $n = 4$). (C) Bradykinin induced a constriction in aortic rings of C57BL/6 mice ($n = 4$) and eNOS^{tg} ($n = 4$) which was dependent on the concentration ($P < 0.0001$, two-way ANOVA) and on the mouse strain ($P < 0.0001$, two-way ANOVA, $*P < 0.05$ vs C57BL/6 mice and eNOS^{tg}, Newman-Keuls test). Inhibition of eNOS by 100 μ M L-NAME added to the organ bath augmented the constriction in C57BL/6 mice ($*P < 0.05$ vs untreated C57BL/6 mice, Newman-Keuls test, $n = 4$) and in eNOS^{tg} ($P < 0.05$ vs untreated eNOS^{tg}, Newman-Keuls test, $n = 4$), while there was no difference between C57BL/6 mice and eNOS^{tg} ($P < 0.05$, Newman-Keuls test, $n = 4$).

bioavailability, and normal mice that underwent pharmacologic treatment to change *in vivo* bioavailability of NO. In addition, C57BL/6 mice were treated with C1-INH to reduce endogenous bradykinin levels or with icatibant to impair binding of bradykinin to B2R. None of these cells and *in vivo* models showed a variation in B2R protein expression in myocardial, lung, and skin tissue and no change of B2R activity in murine endothelial cells, but an NO-dependent augmentation of mouse aortic constriction. This surprising stability of B2R expression suggests that although NO is important for B2R function, it has no effect on B2R activity. Our findings also imply that even repeated therapy with C1-INH or icatibant is unlikely associated with a reduction in their activity.

The data obtained in this investigation are in good agreement with previous reports (13, 14). However, using a reporter gene, it has been shown that stimulation of B2R with bradykinin increases B2R promoter activity in rats, suggesting upregulation of this G protein-coupled receptor upon agonist stimulation (15). In contrast, it was reported recently that the density of B2R labeled with green fluorescent protein and transfected in HEK 293 cells decreased upon prolonged stimulation with inactivation-resistant bradykinin receptor agonists. At the same time, the density of free green fluorescent protein increased (14). These data do not support a mechanism including less transcription and/or less mRNA stability, but point to a posttranslational mechanism consisting of increased endocytosis of B2R and subsequent desensitization and/or degradation. Most likely, this type of regulation occurs in the absence of NO signaling as HEK 293 cells do not express eNOS (16).

In vascular smooth muscle cells, lung fibroblasts, tracheal muscle and, in rats, *in vivo* B2R were shown to be upregulated by interleukin 1 β (17), glucocorticoids (18), tumor necrosis factor α (19), glucose (20), estrogen (21), and platelet-derived growth factor (22). In contrast, there are just few reports on B2R upregulation in endothelial cells including a response to hypoxia and lovastatin (23, 24). These reports described several mechanisms underlying increased B2R gene expression involving the 3'- and the 5'-untranslated region of the B2R gene, the transcription factors p53, KLF-4, and cAMP response element binding protein (CREB), and/or CREB-binding protein (CBP). Among those, phosphorylation of CREB is known to occur after stimulation of vascular smooth muscle cells with NO donors as well. This effect is likely mediated by cGMP-dependent inhibition of phosphodiesterase III and subsequent accumulation of cAMP (25). However, in the present investigation, even concentrations of NO about 1000 times higher than the steady-state concentration of NO in the human vasculature *in vivo* (26, 27) did not increase B2R expression in PAEC and bEND.3. These findings match the results obtained in various mouse models *in vivo*, suggesting that the NO-cGMP signal transduction pathway is likely not involved in B2R gene expression in endothelial cells. The same holds true for highly vascularized murine tissues such as lung, myocardium, and skin.

It has been reported that long-term pharmacologic therapy with C1-INH might increase the attack frequency in several HAE patients (5). Although C1-INH has proven efficacy in this disease and although the clinical experience expand to about 30 years (28, 29), no controlled trial has ever investigated its long-term efficacy and safety. C1-INH inhibits kallikrein, the Hageman factor (factor XII), and plasmin. Kallikrein and the Hageman factor are crucially involved in endogenous formation of bradykinin in a positive feedback loop (30, 31). Therefore, C1-INH inhibits endogenous bradykinin formation and it has been shown previously that human C1-INH in the concentration used in this study strongly reduces bradykinin-induced extravasation in mice (32). Nevertheless, C1-INH had no effect on B2R expression in C57BL/6 mice, suggesting that the magnitude of B2R activation *in vivo* has no impact on B2R expression no matter which B2R signaling pathways are activated. These findings are in line with the observation that icatibant had no effect on B2R expression *in vivo*. Icatibant inhibits B2R signaling and is effective for the treatment of HAE (29) and most likely RAE (33).

The results of our studies on B2R activity in cells and mouse tissues are in good agreement with the expression data discussed above. Monitoring of iCa following stimulation with increasing concentrations of bradykinin demonstrated that NO does not change the increase of iCa²⁺ of bEnd.3 in response to bradykinin. Thus, NO does not appear to modulate G α_q protein-dependent bradykinin signaling (33). As reported previously, C57BL/6 mice abdominal aortic rings respond to bradykinin with a concentration-dependent constriction that is almost completely abolished by icatibant (34). A similar B2R-mediated response could be demonstrated in our experiments with C57BL/6 mice thoracic aorta.

Moreover, bradykinin-induced constriction was completely inhibited by the unselective cyclooxygenase inhibitor diclofenac. This finding suggests that stimulation of B2R in C57BL/6 mice thoracic aorta releases a cyclooxygenase product that mediates vasoconstriction. A similar observation has been made with acetylcholine in hamster aortic rings (35).

We found that the aortic constriction to bradykinin was almost doubled following inhibition of NOS by L-NA or in eNOS^{-/-} mice. Exposure of aortic rings to L-NA increased the vasoconstrictor response to bradykinin to a level comparable with that observed in thoracic aorta of eNOS^{-/-}. Therefore, aortic responses to B2R activation were dependent on eNOS activity inasmuch as generation of NO induced by bradykinin counteracts the vasoconstrictor effect of bradykinin described above. However, these effects were similar in aortic rings of mouse strains with strikingly different bioavailability of vascular NO. This could be demonstrated by a comparable augmentation of aortic constriction following L-NA in C57BL/6 mice and eNOS^{fl}. Thus, the effect of NO- on B2R-induced mouse aortic constriction appears to be limited but this finding does not rule out a role of NO in bradykinin-induced extravasation and/or angioedema.

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Conflicts of interest

GK and MB received an unrestricted educational grant of Shire Deutschland GmbH. GK received lecture fees from Boehringer, Actavis, Mundipharma, and Pfizer and consulting fees from Pfizer. All other authors declare that they have no conflict of interests.

Author contribution

MB, VTD, TS, and GK contributed to the conception of the study. FK, TS, VTD, OK, FFH, and SV conducted the experiments and the corresponding data analysis. NB and MK assisted with the NOVostar[®] plate reader studies and the corresponding data analysis. GK supervised the project and wrote the manuscript. FK, MB, and TS reviewed and edited the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Full blots showing the staining pattern of the anti-B2R antibodies used in this study in bEND.3 (A,C) and PAEC (B,D) with or without a 3 h incubation with 10 μ M DEA/NO. Shown are the staining pattern of (A) the mouse monoclonal BD anti-B2R antibody in bEND.3 and (B)

PAEC and the staining pattern of the rabbit monoclonal Abcam anti-B2R antibody in (C) bEND.3 and (D) PAEC.

Figure S2. Full blots showing the staining pattern of the anti-B2R antibodies used in this study in (A,B) lung and (C) dorsal skin tissue homogenates of C57BL/6, eNOS^{-/-} and B2R^{-/-}.

Data S1. Validation of B2R antibodies. The mouse monoclonal BD antibody binds to nonglycosylated B2R and should

show a double band at about 42 kD, while the rabbit monoclonal Abcam antibody binds to glycosylated B2R and should show a single band at about 78 kD. Surprisingly, the signal given by the BD antibody in cell homogenates appeared rather below 42 kD, while the Abcam antibody gave the expected band.

References

- Bas M, Hoffmann TK, Kojda G. Evaluation and management of angioedema of the head and neck. *Curr Opin Otolaryngol Head Neck Surg* 2006;**14**:170–175.
- Agostoni A, Cicardi M. Drug-induced angioedema without urticaria. *Drug Saf* 2001;**24**:599–606.
- Kelemen Z, Moldovan D, Mihály E, Visy B, Széplaki G, Csuka D et al. Baseline level of functional C1-inhibitor correlates with disease severity scores in hereditary angioedema. *Clin Immunol* 2010;**134**:354–358.
- Fox AJ, Laloo UG, Belvisi MG, Bernareggi M, Chung KF, Barnes PJ. Bradykinin-evoked sensitization of airway sensory nerves: a mechanism for ACE-inhibitor cough. *Nat Med* 1996;**2**:814–817.
- Bork K, Hardt J. Hereditary angioedema: increased number of attacks after frequent treatments with C1 inhibitor concentrate. *Am J Med* 2009;**122**:780–783.
- Higashi Y, Sasaki S, Nakagawa K, Ueda T, Yoshimizu A, Kurisu S et al. A comparison of angiotensin-converting enzyme inhibitors, calcium antagonists, beta-blockers and diuretic agents on reactive hyperemia in patients with essential hypertension: a multicenter study. *J Am Coll Cardiol* 2000;**35**:284–291.
- O'Driscoll G, Green D, Maiorana A, Stanton K, Coleavay F, Taylor R. Improvement in endothelial function by angiotensin-converting enzyme inhibition in non-insulin-dependent diabetes mellitus. *J Am Coll Cardiol* 1999;**33**:1506–1511.
- Koh KK, Bui MN, Hathaway L, Csako G, Wacławiw MA, Panza JA et al. Mechanism by which quinapril improves vascular function in coronary artery disease. *Am J Cardiol* 1999;**83**:327–331.
- Shesely EG, Maeda N, Kim HS, Desai KM, Kregel JH, Laubach VE et al. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 1996;**93**:13176–13181.
- Suvorava T, Lauer N, Kumpf S, Jacob R, Meyer W, Kojda G. Endogenous vascular hydrogen peroxide regulates arteriolar tension in vivo. *Circulation* 2005;**112**:2487–2495.
- Oppermann M, Balz V, Adams V, Dao VT, Bas M, Suvorava T et al. Pharmacologic induction of vascular extracellular superoxide dismutase expression in-vivo. *J Cell Mol Med* 2009;**13**:1271–1278.
- Bawolak MT, Fortin S, Bouthillier J, Adam A, Gera L, C-Gaudreault R et al. Effects of inactivation-resistant agonists on the signaling, desensitization and down-regulation of bradykinin B(2) receptors. *Br J Pharmacol* 2009;**158**:1375–1386.
- Pesquero JB, Lindsey CJ, Paiva AC, Ganten D, Bader M. Transcriptional regulatory elements in the rat bradykinin B2R receptor gene. *Immunopharmacology* 1996;**33**:36–41.
- Schmidt K, Andrew P, Schrammel A, Groschner K, Schmitz V, Kojda G et al. Comparison of neuronal and endothelial isoforms of nitric oxide synthase in stably transfected HEK 293 cells. *Am J Physiol Heart Circ Physiol* 2001;**281**:H2053–H2061.
- Schmidlin F, Scherrer D, Daeflter L, Bertrand C, Landry Y, Gies JP. Interleukin-1beta induces bradykinin B2R receptor gene expression through a prostanoid cyclic AMP-dependent pathway in human bronchial smooth muscle cells. *Mol Pharmacol* 1998;**53**:1009–1015.
- Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation* 2001;**103**:2839–2844.
- Enquist J, Skroder C, Whistler JL, Leeb-Lundberg LM. Kinins promote B2R receptor endocytosis and delay constitutive B1 receptor endocytosis. *Mol Pharmacol* 2007;**71**:494–507.
- Scherrer D, Schmidlin F, Haddad EB, Kasel O, Landry Y, Gies JP. Glucocorticoids increase bradykinin B2R receptor gene transcription in cultured guinea-pig tracheal smooth muscle cells. *Naunyn Schmiedeberg Arch Pharmacol* 1999;**359**:153–159.
- Haddad EB, Fox AJ, Rousell J, Burgess G, McIntyre P, Barnes PJ et al. Post-transcriptional regulation of bradykinin B1 and B2R receptor gene expression in human lung fibroblasts by tumor necrosis factor-alpha: modulation by dexamethasone. *Mol Pharmacol* 2000;**57**:1123–1131.
- Christopher J, Velarde V, Zhang D, Mayfield D, Mayfield RK, Jaffa AA. Regulation of B(2)-kinin receptors by glucose in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2001;**280**:H1537–H1546.
- Madeddu P, Emanuelli C, Song Q, Varoni MV, Demontis MP, Anania V et al. Regulation of bradykinin B2R-receptor expression by oestrogen. *Br J Pharmacol* 1997;**121**:1763–1769.
- Dixon BS, Sharma RV, Dennis MJ. The bradykinin B2R receptor is a delayed early response gene for platelet-derived growth factor in arterial smooth muscle cells. *J Biol Chem* 1996;**271**:13324–13332.
- Liesmaa I, Leskinen HK, Kokkonen JO, Ruskoaho H, Kovanen PT, Lindstedt KA. Hypoxia-induced expression of bradykinin type-2 receptors in endothelial cells triggers NO production, cell migration, and angiogenesis. *J Cell Physiol* 2009;**221**:359–366.
- Liesmaa I, Kokkonen JO, Kovanen PT, Lindstedt KA. Lovastatin induces the expression of bradykinin type 2 receptors in cultured human coronary artery endothelial cells. *J Mol Cell Cardiol* 2007;**43**:593–600.
- Steinbicker AU, Liu H, Jiramongkolchai K, Malhotra R, Choe EY, Busch CJ et al. Nitric oxide regulates pulmonary vascular smooth muscle cell expression of the inducible cAMP early repressor gene. *Nitric Oxide* 2011;**25**:294–302.
- Kojda G, Patzner M, Hacker A, Noack E. Nitric oxide inhibits vascular bioactivation of glyceryl trinitrate. A novel mechanism to explain preferential venodilation of organic nitrates. *Mol Pharmacol* 1998;**53**:547–554.
- Vallance P, Patton S, Bhagat K, MacAllister R, Radomski M, Moncada S et al. Direct measurement of nitric oxide in human beings. *Lancet* 1995;**346**:153–154.
- Bork K, Barnstedt SE. Treatment of 193 episodes of laryngeal edema with C1 inhibitor concentrate in patients with hereditary angioedema. *Arch Intern Med* 2001;**161**:714–718.
- Cicardi M, Banerji A, Bracho F, Malbrán A, Rosenkranz B, Riedl M et al. Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema. *N Engl J Med* 2010;**363**:532–541.
- Kaplan AP. Enzymatic pathways in the pathogenesis of hereditary angioedema: the

- role of C1 inhibitor therapy. *J Allergy Clin Immunol* 2010;**126**:918–925.
31. Kaplan AP, Joseph K. The bradykinin-forming cascade and its role in hereditary angioedema. *Ann Allergy Asthma Immunol* 2010;**104**:193–204.
32. Han ED, MacFarlane RC, Mulligan AN, Scafidi J, Davis AE III. Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. *J Clin Invest* 2002;**109**:1057–1063.
33. Bas M, Greve J, Stelter K, Bier H, Stark T, Hoffmann TK et al. Therapeutic efficacy of icatibant in angioedema induced by angiotensin-converting enzyme inhibitors: a case series. *Ann Emerg Med* 2010;**56**:278–282.
34. Felipe SA, Rodrigues ES, Martin RP, Paiva AC, Pesquero JB, Shimuta SI. Functional expression of kinin B1 and B2R receptors in mouse abdominal aorta. *Braz J Med Biol Res* 2007;**40**:649–655.
35. Wong SL, Leung FP, Lau CW, Au CL, Yung LM, Yao X et al. Cyclooxygenase-2-derived prostaglandin F2alpha mediates endothelium-dependent contractions in the aortae of hamsters with increased impact during aging. *Circ Res* 2009;**104**:228–235.

Supplementary Information

Stability of murine bradykinin type 2 receptor despite treatment with NO, bradykinin, icatibant or C1-INH.

Farbod Khosravani¹, PharmD

Tatsiana Suvorava¹, PhD

Vu Thao-Vi Dao¹, PharmD

Nicole Brockmann², PharmD

Oktay Kocgirli¹, MD vet

Frederick Franziskus Herbst, PharmD

Sandra Valcaccia¹, MD

Matthias U. Kassack², PhD,

Murat Bas³, MD,

Georg Kojda PharmD¹, PhD*

¹Institute of Pharmacology and Clinical Pharmacology, Heinrich Heine University, Düsseldorf, Germany, ²Institute of Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University, Düsseldorf, Germany, ³Otorhinolaryngology Department, University Hospital Rechts der Isar, Munich Technical University, Munich, Germany

Supplementary Information

Methods and Materials

Mice. Mice were housed at 22°C in the animal facility of the university at a 12 h light-dark cycle and received water and food ad libitum. PETN (PETN, a generous gift of Actavis GmbH, Monheim, Deutschland) was incorporated in the food pellets in two different concentrations to achieve the desired daily dose (1). Previous studies have shown that this procedure resulted in measurable plasma concentrations of PETN metabolites that correlated with the dose given (2). N^o-nitro-L-arginine (L-NA, Sigma, Germany) was given with the drinking water (pH 3) in a concentration of 1,5 mg/l for 3 weeks before the experiments. Given that the mean intake of water is 2 ml this concentration translates to a dose of 100 mg L-NA/kg BW/day. Previous studies have shown that this dose is sufficient to achieve an increase in blood pressure that matches that of eNOS^{-/-} (3). All mice were monitored for infections and other diseases every other day and only healthy mice were included in the study experiments.

Cell Culture Porcine aortic endothelial cells (PAEC) were isolated from fresh porcine thoracic aortae and murine brain endothelial cells (bEnd.3) cells were purchased from ATCC (Teddington, Middlesex, UK). Cells were grown to confluence within a week to 10 days and cultured as described previously (4). For all experiments cells of passage 2-3 were used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA) and 100 units/ml of penicillin/streptomycin (Sigma, St. Louis, MO, USA), at 37°C in the presence of 5% CO₂. Cells of passages 24-28 were used.

Cell Incubation Experiments Standard media was replaced by media without fetal bovine serum and cells were subjected to the NO-donor DEA/NO for 3 h and the long-acting NO donor DETA/NO (100 µM) for up to 24 h. After the incubation cells were scraped and

homogenized in lysis buffer by freezing, thawing and subsequent subjection to ultrasound for 15 min at 4°C.

Western blotting. Western blot analysis was done in a blinded fashion as described previously (5) using commercially available mouse monoclonal antibodies directed against bradykinin type 2 receptor (B2R) and eNOS (BD Transduction Laboratories, Lexington, Kentucky, USA) and a rabbit monoclonal anti B2R antibody (Abcam, Cambridge, UK). Total protein levels were determined by the Bradford method (6).

Real-Time Reverse Transcription-PCR Total RNA from bEND.3 was isolated using the QIAshredder and RNeasy kit (Qiagen, Hilden, Germany), including an on-column DNase digestion. Complementary DNA was synthesized from 1 µg total RNA by TurboTM DNase (Ambion, Carlsbad, USA) according to the manufacturers' protocol. Real time RT-PCR was performed with TaqMan[®] Gene Expression Assays (Applied Biosystems, Weiterstadt Germany) using Mm01339967_m1 for mouse AT2 detection, respectively. For normalization purpose, co-amplification of hypoxanthine guanine phosphoribosyl transferase (hprt1) cDNA was performed with the TaqMan[®] Gene Expression Assay for mouse hprt1 (Mm00446968). Real time RT-PCR was carried out on a 7300 Real-Time PCR System (Applied Biosystems, Weiterstadt Germany). B2R mRNA expression levels relative to hprt1 were determined using the $\Delta\Delta C_t$ method and expressed in arbitrary units.

Ca²⁺ measurements in bEnd.3 cells. Cells (20.000-35.000 cells/well) were loaded with a membrane permeable fluorescent dye (Oregon Green 488 BAPTA-1AM, Molecular Probes, Eugene, OR) and Ca²⁺ fluorescence measurements were performed by using a NOVOstar[®] plate reader with a pipette system (BMG labTechnologies, Offenburg, Germany) and evaluated using the NOVOstar[®]-software (7). Concentration response curves were performed by adding 10 nM – 1 mM bradykinin following treatment with vehicle, icatibant (30 min) or DEA/NO (3 h).

Aortic reactivity studies Function of the endothelium was examined by cumulative addition of acetylcholine (Ach, 0.01 to 10 μ M) as described previously (8). Bradykinin (0.01 to 10 μ M) was added by cumulative application after precontraction with phenylephrine (0.2 μ M) in the presence or absence of endothelium or L-NA (100 μ M). Some preparations were preincubated with the B2R-antagonist icatibant (former Hoe-140, 10 μ M) and with the non-selective COX-inhibitor diclofenac (10 μ M).

Materials If not otherwise indicated all chemicals were obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany), Merck KGaA (Darmstadt, Germany) or Carl Roth GmbH + Co. KG (Karlsruhe, Germany).

Statistics. All data were analyzed by standard computer programs (GraphPad Prism PC Software, Version 3.0, Analysis of Variance, ANOVA) and are expressed as mean values and standard error of the mean (S.E.M.). Significant differences were evaluated using either two-way ANOVA (concentration-response curves), Newman-Keuls Multiple Comparison Test following One Way-ANOVA (multiple comparisons) or students t-test or. A P-value below 0.05 was considered as significant.

Supplementary Results

Validation of B2R antibodies

Several experiments were carried out to evaluate the specificity of both B2R antibodies for binding to B2R in cells and murine tissues. The mouse monoclonal BD antibody binds to non-glycosylated B2R and should show a double band at about 42 kD, while the rabbit monoclonal Abcam antibody binds to glycosylated B2R and should show a single band at about 78 kD. Incubation of bEND.3 with 10 μ M DEA/NO for 3 h did not change the signal given by the BD antibody (Fig. S1A) and similar results were obtained in PAEC (Fig. S1B) and in bEND.3 (Fig. S1C) using the Abcam antibody. In contrast, the Abcam antibody does not give a 78 kD signal in PAEC (Fig. S1D) indicating a variation of species specificity. Nevertheless, both antibodies showed comparable results in bEND.3.

Surprisingly, the signal given by the BD antibody in cell homogenates appeared rather below 42 kD (Fig. S1A,B) and this result could be confirmed in lung tissue of C57BL/6, eNOS^{-/-} and B2R^{-/-} using a ladder including a 40 kD protein. Fig. S2A and Fig. S2C show the results obtained with both antibodies using the same blot, respectively, which was cut at 55 kD. The blots shown in the middle panels of Fig. S2A and Fig. S2C were stripped and stained with a mouse monoclonal anti-gapdh or anti actin antibody to control for protein load (lower panel). While the Abcam antibody showed the expected signal at 78 kD (upper panel), the BD antibody showed the double band clearly below 40 kD (approximately 38 kD for the lower part of the double band, middle panel) which matches the observation in cells. However, the B2R signals of both antibodies (upper panel and middle panel) were strongly reduced in lung homogenates of B2R^{-/-} indicating a large but not complete specificity for B2R.

Importantly, the mouse monoclonal BD antibody shows a strongly intensified signal in eNOS^{-/-} which was evident in each of the samples and rather indicates unspecific staining caused by binding of the secondary anti-mouse IgG antibody. Fig S2B shows the result of a

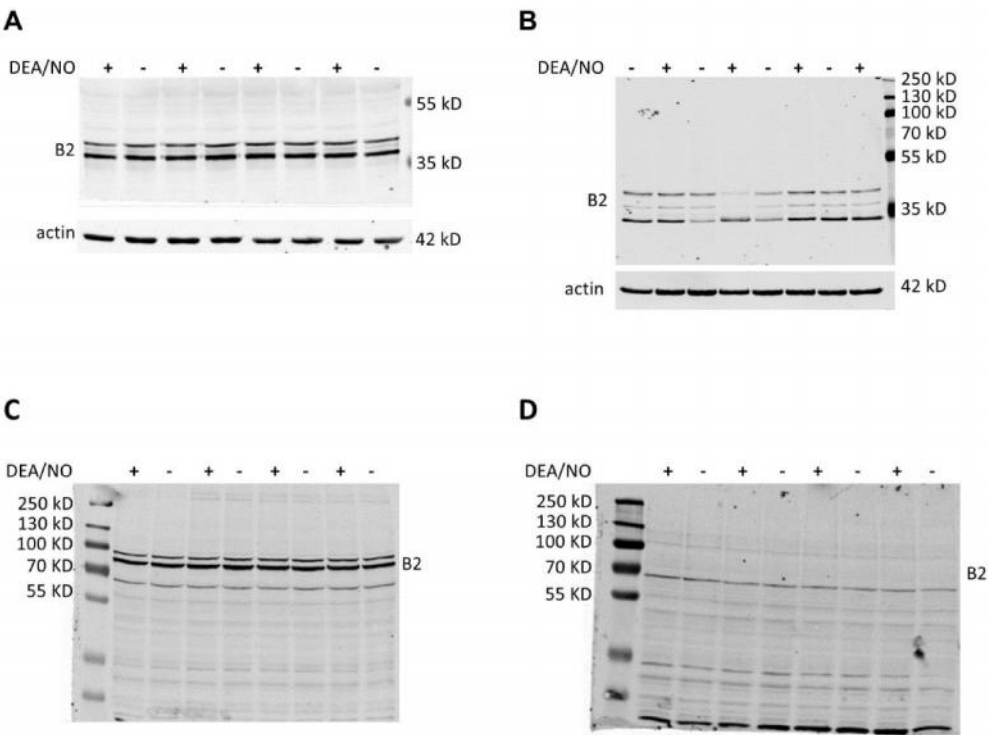
control experiment in which the BD antibody was omitted and the blot was incubated with anti-mouse IgG antibody only. However, such magnification did not occur in homogenates of dorsal skin tissue (Fig S2C). These results suggest that the use of the BD antibody in lung tissue of eNOS^{-/-} is inappropriate. Of note, such a false staining did not occur in lung tissue of B2R^{-/-} mice (Fig. S2A).

Supplementary Figure Legends

Supplementary Figure 1: Full blots showing the staining pattern of the anti-B2R antibodies used in this study in bEND.3 (**A, C**) and PAEC (**B, D**) with or without a 3 h incubation with 10 μ M DEA/NO. Shown are the staining pattern of (**A**) the mouse monoclonal BD anti-B2R antibody in bEND.3 and (**B**) PAEC and the staining pattern of the rabbit monoclonal Abcam anti-B2R antibody in (**C**) bEND.3 and (**D**) PAEC. Cells were pretreated for 3 h with vehicle or DEA/NO as indicated. These blots were performed to evaluate the specificity of the anti-B2R antibodies and the results were not quantified and included in the final results.

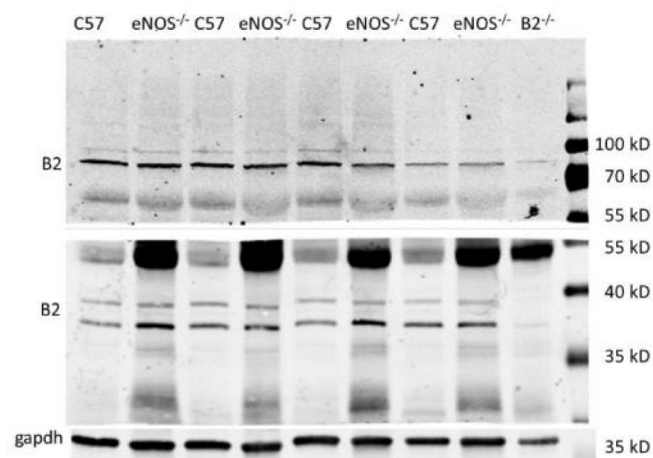
Supplementary Figure 2: Full blots showing the staining pattern of the anti-B2R antibodies used in this study in (**A, B**) lung and (**C**) dorsal skin tissue homogenates of C57BL/6, eNOS^{-/-} and B2R^{-/-}. (**A**) Shown are the staining pattern with the rabbit monoclonal Abcam anti-B2R antibody (upper panel), the mouse monoclonal BD anti-B2R antibody (middle panel). The blot was cut at 55 kD and the part shown in the middle panel was stripped to control for protein load. (**B**) Shown is the staining pattern the anti-mouse IgG antibody only using another blot (the rabbit IgG antibody showed no signal, blot not shown). These blots were performed to evaluate the specificity of the anti-B2R antibodies and the results were not quantified and included in the final results. (**C**) Shown are the staining pattern with the rabbit monoclonal Abcam anti-B2R antibody (upper panel), the mouse monoclonal BD anti-B2R antibody (middle panel). The blot was cut at 55 kD and the part shown in the middle panel was stripped to control for protein load. These blots were quantified and included in the final results.

Supplementary Figure 1

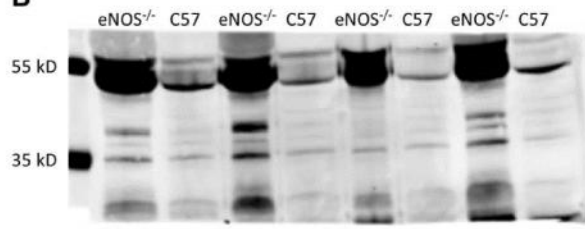


Supplementary Figure 2

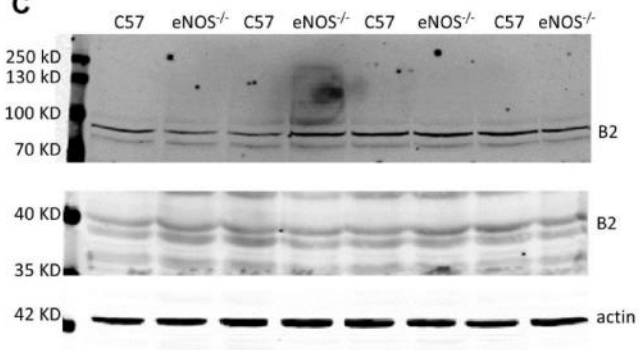
A



B



C



References

1. Oppermann M, Suvorava T, Freudemberger T, et al. Regulation of vascular guanylyl cyclase by endothelial nitric oxide-dependent posttranslational modification. *Basic Res Cardiol* 2011; 106:539-49.
2. Oppermann M, Balz V, Adams V, et al. Pharmacologic Induction of Vascular Extracellular Superoxide Dismutase Expression in-vivo. *J Cell Mol Med* 2009; 13:1271-8.
3. Kojda G, Laursen JB, Ramasamy S, et al. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res* 1999; 42:206-13.
4. Chamley JH, Campbell GR, McConnell JD, Groschel-Stewart U. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. *Cell Tissue Res* 1977; 177:503-22.
5. Kojda G, Cheng YC, Burchfield J, Harrison DG. Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene. *Circulation* 2001; 103:2839-44.
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248-54.
7. Kassack MU, Hofgen B, Lehmann J, Eckstein N, Quillan JM, Sadee W. Functional screening of G protein-coupled receptors by measuring intracellular calcium with a fluorescence microplate reader. *J Biomol Screen* 2002; 7:233-46.
8. Suvorava T, Lauer N, Kojda G. Physical inactivity causes endothelial dysfunction in healthy young mice. *J Am Coll Cardiol* 2004; 44:1320-7.