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Role of vascular Kinin B_1 and B_2 receptors in endothelial nitric oxide metabolism

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ABSTRACT

Kinin B_1 and B_2 receptors play an essential role in inflammatory process and cardiovascular homeostasis. The present study investigated the vascular reactivity and nitric oxide (NO) generation in the isolated mesenteric arteriolar bed from B_1 ($B_1^{-/-}$) and B_2 receptor ($B_2^{-/-}$) knockout mice. Endothelial-dependent relaxation was significantly decreased in arterioles from both $B_1^{-/-}$ and $B_2^{-/-}$ in comparison to wild type (WT) mice, with no differences for endothelial-independent relaxating or vasoconstrictor agents. Plasmatic and vascular NO production were markedly reduced in both $B_1^{-/-}$ and $B_2^{-/-}$. In contrast, in the presence of L-arginine, Ca^{2+} and co-factors for the enzyme, NO synthase activity was higher in homogenates of mesenteric vessels of $B_1^{-/-}$ and $B_2^{-/-}$. The present study demonstrated that targeted deletion of B_1 or B_2 receptor gene in mice induces important alterations in the vascular reactivity of resistance vessels and NO metabolism. The severe impairment in the endothelial-mediated vasodilation accompanied by decreased NO bioavailability, despite the augmented NOS activity, strongly indicates an exacerbation of NO inactivation in $B_1^{-/-}$ and $B_2^{-/-}$ vessels. The present data provide valuable information in order to clarify the relevance of kinin receptors in regulating vascular physiology and may point to new approaches regarding its correlation with endothelial dysfunction, oxidative stress and NO availability.

1. Introduction

The kallikrein–kinin system plays an important role in several biological functions, including inflammation and cardiovascular homeostasis [7]. The diverse range of effects elicited by kinins is mediated by activation of G protein-coupled receptors, named B_1 and B_2. Bradykinin (BK) is the natural agonist of the B_2 receptor, and its degradation by carboxypeptidases generates the B_1 receptor agonist, des-Arg[9]-BK [34]. Whereas B_2 receptors are constitutively expressed and mediate most of the known effects assigned to kinins, B_1 receptors are weakly detectable under physiological conditions, but rapidly induced by inflammatory stimuli [7,23].

Both B_1 and B_2 receptors act through $G\alpha_q$ to stimulate phospholipase Cβ followed by phosphoinositide hydrolysis and intracellular free Ca^{2+} mobilization [19]. The resulting intracellular free Ca^{2+} is the initial step in the activation of nitric oxide synthase (NOS), which catalyzes oxidation of the terminal guanidine nitrogen of L-arginine to form L-citrulline and nitric oxide (NO) [32]. Three NOS isoforms have been described: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The iNOS isoform differs from nNOS and eNOS in that it is fully active in the absence of Ca^{2+} [27]. The NOS isoforms have similar enzymatic mechanisms and require presence of co-factors tetrahydrobiopterin (BH_4), nicotinamide-adenine dinucleotide (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) for its proper function [25]. In the vasculature, once formed by NOS, endothelial NO diffuses in to the smooth muscle and activates soluble guanylate cyclase that catalyzes the formation of 3′,5′-cyclic guanosine monophosphate (cGMP), resulting in smooth muscle relaxation and therefore vasodilation [13].

In the last recent years, the development of genetically engineered mice lacking kinin receptors has allowed a better understanding of the physiological and pathological role of the kallikrein–kinin system in a wide range of biological events [31]. Mice with a targeted deletion of the gene for the B_1 receptor ($B_1^{-/-}$) are described to be healthy, fertile and normotensive, but exhibit blunted responses to bacterial lipopolysaccharide injection and hypoalgesia [30]. Under physiological conditions, B_2 receptor knockout mice ($B_2^{-/-}$) present normal development [9], renal hemodynamics and salt balance [2,26,35]. Nevertheless, data regarding the effects of B_2 receptor deletion on blood pressure regulation are controversial. Some authors have demonstrated that $B_2^{-/-}$ are normotensive [1–3,11,12,26,35,37,39] while other groups

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observed a slight but significant increase in blood pressure levels [15,16,21,22].

Considering that both B_1 and B_2 receptors are located in the endothelium and in vascular smooth muscle cells [7,19], and that resistance vessels are the most important sites for determining peripheral vascular resistance [38], the present study was addressed to investigate the vascular reactivity of mesenteric arterioles of $B_1^{-/-}$ and $B_2^{-/-}$ in response to endothelium-dependent and -independent agonists. In parallel, plasma NO levels, vascular NO release and NOS activity in the mesenteric vessels were also analyzed in order to provide information about NO bioavailability in these mice strains.

2. Methods

2.1. Animals

C57Bl/6 male knockout B_1 ($B_1^{-/-}$), B_2 ($B_2^{-/-}$) and wild type (WT) mice, aged 10–14 weeks were obtained from the breeding stock of Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME – UNIFESP). Mice were kept in a temperature-controlled room on a 12 h light/day cycle, 60% humidity, standard mice chow and water *ad libitum*. In $B_1^{-/-}$ and $B_2^{-/-}$, the absence of the kinins receptors was shown by undetectable level of mRNA encoding for the B_1 or B_2 receptor, respectively, using a semi-quantitative RT-PCR technique. All procedures were approved and performed in accordance with the guidelines of the Ethics Committee of the UNIFESP (protocol number 0928/05), conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Vascular reactivity in the perfused mesenteric vascular bed

Isolated mesenteric vascular beds were prepared as previously described for the rat preparation [24], with slight adaptations for the mouse. The mesenteric vascular bed was perfused with Krebs-Henseleit solution, pH 7.4, 37 °C, gassed with 95% O_2 and 5% CO_2, at a constant rate of 2 mL/min using a peristaltic pump. Vascular responses were evaluated by changes in the perfusion pressure (mmHg) measured by a data acquisition system (PowerLab 8/S, ADInstruments Pty Ltda, Australia). To confirm the viability of tissues, preparations were perfused with KCl (90 mmol/L) added to the Krebs solution for 5 min. After 30 min of stabilization, increasing doses of norepinephrine (NE) (5–100 nmol), acetylcholine (ACh) (0.1–10 nmol) and sodium nitroprusside (SNP) (0.1–10 nmol) were injected *in bolus*, in a volume range of 30–100 μL, with a 3-min interval between each dose. For ACh and SNP assays, preparations were pre-contracted with NE (10 μmol/L) added to Krebs solution and vascular responses were calculated as percentage of contraction induced by NE.

2.3. Measurements of circulating NO levels

The plasma NO levels were evaluated by NO derivatives nitrate and nitrite, as previously described [28]. Blood samples were collected into EDTA-coated tubes and plasma was immediately separated by low-speed centrifugation (1500 × *g*). The concentration of nitrate in blood was determined by chemiluminescence, elicited by the reaction of NO with ozone after nitrate reduction with VCl_3 saturated solution in 1 mol/L HCl, at 90 °C, using a NO analyzer (NOA^{TM280} Sievers Instruments Inc., Boulder, CO, USA). Nitrite was determined after reduction with 1% KCl solution in glacial acetic acid to convert nitrite to NO.

2.4. Basal NO production in mesenteric arterioles

Basal NO in mesenteric arterioles was determined by using a fluorescent cell permeable dye for NO, 4,5 diaminofluorescein diacetate (DAF-2 DA, Alexis, USA), as previously described [10]. Once inside the cell, DAF-2 DA is hydrolyzed by cytosolic esterases thus releasing DAF-2. The reaction between DAF-2 and NO yields the corresponding bright green-fluorescent triazolofluoresceins (DAF-2T). The mesenteric arterioles were dissected, immersed in medium for cryosectioning and cut into 10 μm thick sections (Leica CM 1850 cryostat, Leica Instruments, Germany). In order to stimulate NOS activation and provide optimal levels of substrate, slices were pre-incubated with phosphate buffer (PB) solution containing $CaCl_2$ (0.45 μmol/L) and L-arginine (100 μmol/L) during 30 min at 37 °C. Slices were washed, incubated with PB containing DAF-2 DA (10 μmol/L) for 30 min at 37 °C and observed on a microscope (Axiovert 100 M – Carl Zeiss SMT, Germany) equipped with fluorescein filter (excitation at 488 nm and measuring emission at 515 nm). Fluorescence emitted in response to NO production was quantified through optic densitometry (arbitrary units, a.u.) using the AxioVision 4.8. digital images analysis software (Carl Zeiss). The semi-quantitative analysis of basal NO production was determined, at least, in three slices from each animal. Significant auto-fluorescence was discarded by experiments performed in the absence of DAF-2DA.

2.5. Detection of NOS activity in the mesenteric vascular bed

NOS activity was measured by the biochemical conversion of L-[3H] arginine to L-[3H] citrulline according to the method described by Rees et al. [33]. Mesenteric vessels were dissected, washed, homogenized in ice-cold buffer and stored at −80 °C. On the day of assay, homogenates were incubated (37 °C/60 min) in a buffer containing FMN and FAD 4 μmol/L, calmodulin 10 μg/mL, Ca^{2+} 1.25 mmol/L, NADPH 1 mmol/L, L-arginine 120 nmol/L, L-[3H] arginine 50 nmol/L (NEN Life Science Products, USA) and BH_4 10 μmol/L. For the determination of iNOS activity, experiments were performed in the absence of Ca^{2+}. Reaction was terminated by the addition of cation-exchange resin (Dowex 50WX8-400) to remove the excess of substrate. The resin was left to settle for 30 min at room temperature, the supernatant was carefully removed in vials with scintillation liquid and the radioactivity to L-[3H] citrulline was quantified. Results were normalized by protein concentration and NO synthase activity was expressed as pmol/mg min.

2.6. Drugs and reagents

NE, ACh and SNP were acquired from Sigma Chemical Co. (St. Louis, MO). Except when described, all other drugs and reagents were purchased from Merck, Sharp & Döhme (Whitehouse Station, NJ).

2.7. Statistical analysis

Comparisons were made by ANOVA followed by Tukey–Kramer test. Values were reported as mean ± standard error of mean (SEM). Statistical significance was set as $P < 0.05$.

3. Results

3.1. Vascular reactivity to NE, ACh and SNP

After 30 min of stabilization, basal perfusion pressure in mesenteric vascular bed from $B_2^{-/-}$ (48 ± 1.8 mmHg; $n = 8$; $P < 0.05$) was significantly higher when compared to WT (40 ± 1.4 mmHg; $n = 11$)

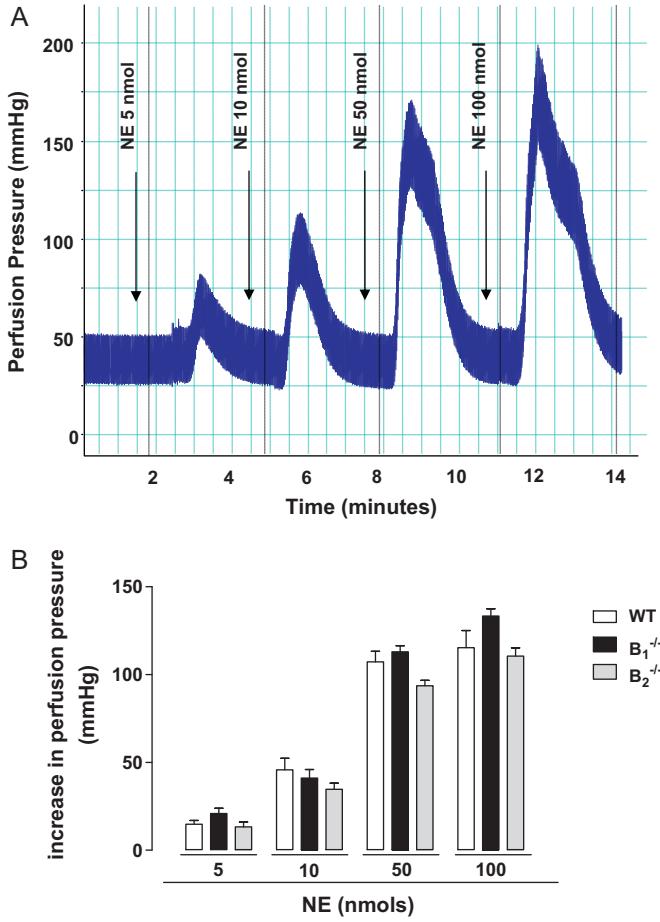

Fig. 1. (A) Representative recording of perfusion pressure alteration elicited by *in bolus* injection of NE in the mice isolated mesenteric arteriolar bed. Arrows indicate drug application. (B) Bars graph shows the vasoconstrictor effect of NE (determined as increase in perfusion pressure) on isolated mesenteric arteriolar bed of WT, $B_1^{-/-}$ and $B_2^{-/-}$. Results are mean ± S.E.M., $n = 5–7$ for each group.

and $B_1^{-/-}$ (41 ± 1.0 mmHg; $n = 8$) preparations. Injection of vasoconstrictor NE on isolated vascular preparations elicited rapid and dose-related constriction that increased to a single peak and then declined to basal perfusion pressure, usually within 2 min (Fig. 1A). NE injection promoted similar responses in all vascular preparations from WT, $B_1^{-/-}$ and $B_2^{-/-}$, as demonstrated in Fig. 1B.

The endothelial function of mesenteric arterioles was assessed through the effect of ACh (an endothelium-dependent relaxating agent) and SNP (an endothelium-independent relaxating agent) in pre-contracted vessels (NE 10 μmol/L). In all experiments, ACh produced a significant dose-dependent reduction in perfusion pressure (at the doses of 0.1, 1 and 10 nmols). As shown in Fig. 2, vascular response to ACh was markedly reduced in $B_1^{-/-}$ and $B_2^{-/-}$ preparations when compared to WT responses, for all tested doses. In all groups, SNP injection elicited a consistent decrease in perfusion pressure (about 60% of contraction induced by NE perfusion at the dose of 10 nmols). No significant differences were detected among strains for all tested doses of SNP (Fig. 3).

3.2. Plasmatic NO levels

Since the NO metabolites reflect the overall NO production in the organism, we determined the plasma nitrite/nitrate concentration in blood samples obtained from WT, $B_1^{-/-}$ and $B_2^{-/-}$ mice. A significant decrease in circulating NO levels was detected in both $B_1^{-/-}$ and $B_2^{-/-}$ when compared to WT samples. Data are shown in Fig. 4.

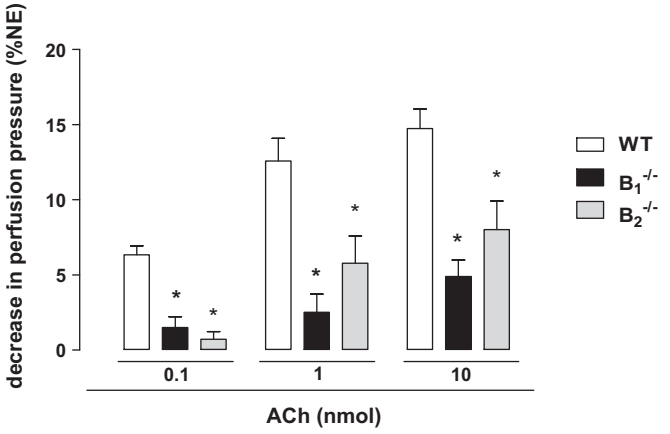

Fig. 2. Bars graph shows the relaxating effect of ACh in isolated mesenteric arteriolar bed of WT, $B_1^{-/-}$ and $B_2^{-/-}$. Responses are expressed as % of contraction induced by NE (10 μmol/L). Results are mean ± S.E.M., $n = 5–7$ for each group. *$P < 0.05$ *vs* WT.

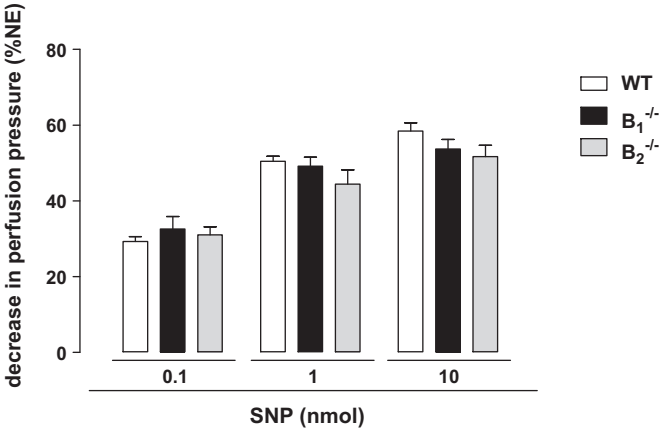

Fig. 3. Bars graph shows the relaxating effect of SNP in isolated mesenteric arteriolar bed of WT, $B_1^{-/-}$ and $B_2^{-/-}$. Responses are expressed as % of contraction induced by NE (10 μmol/L). Results are mean ± S.E.M., $n = 6–7$ for each group.

3.3. Basal NO production in mesenteric arterioles

Vascular NO production was assessed in mesenteric arterioles sections incubated with DAF-2 DA, a sensitive fluorescent indicator for detection of NO. Images are shown in Fig. 5A. The fluorescence

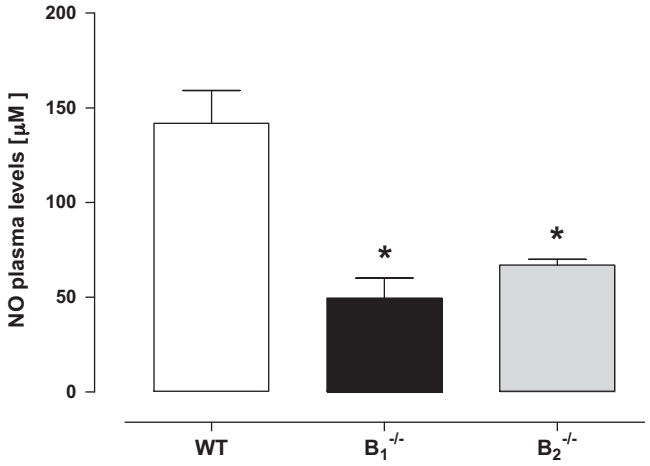

Fig. 4. Bars graph shows the NO plasma levels of WT, $B_1^{-/-}$ and $B_2^{-/-}$. Results are mean ± S.E.M., $n = 4–5$ for each group. *$P < 0.05$ *vs* WT.

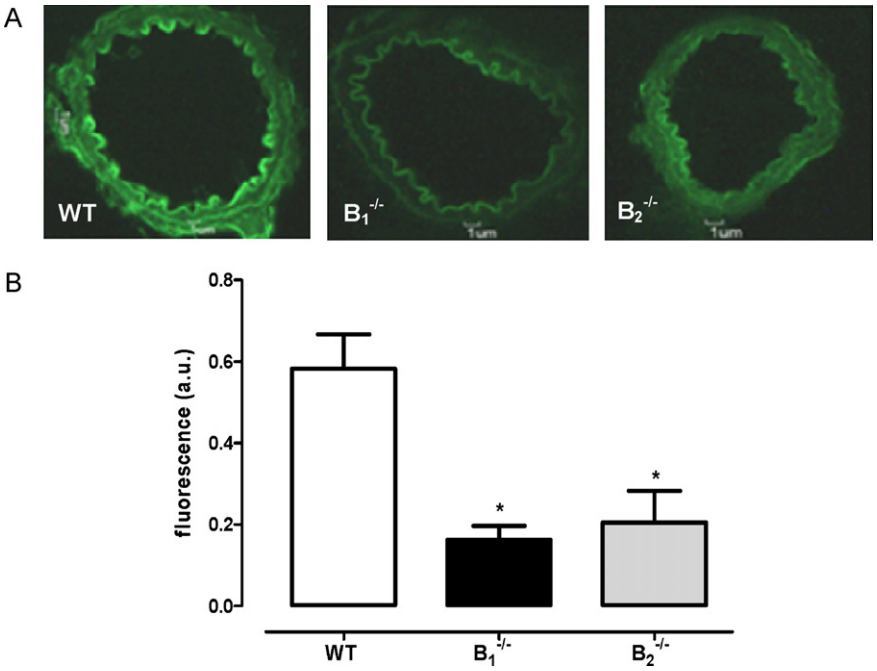

Fig. 5. (A) Representative images of fluorescence in mesenteric arterioles slices from WT, $B_1^{-/-}$ and $B_2^{-/-}$ after incubation with DAF-2DA (10 μmol/L), a fluorescent cell permeable dye for NO (200× magnification). (B) Bars graph shows the fluorescence emitted after incubation with DAF-2DA and represent the basal NO production. Fluorescence was quantified by optic densitometry (arbitrary units, a.u.). Results are mean ± S.E.M., $n = 5$ for each group. $^*P < 0.05$ *vs* WT.

intensity of DAF-2 DA was significantly diminished in vessels from $B_1^{-/-}$ and $B_2^{-/-}$ when compared to WT samples, indicating that basal NO production was decreased in mesenteric arterioles from both strains (Fig. 5B).

3.4. NOS activity in mesenteric vessels

The NOS activity was assessed in homogenates of mesenteric vessels by biochemical conversion of L-[3H] arginine to L-[3H] citrulline in presence of substrate and co-factors. Surprisingly, instead of the expected reduction, total NOS activity (Ca^{2+}-dependent) was enhanced in mesenteric vessels from $B_1^{-/-}$ and $B_2^{-/-}$ when compared with WT samples, as represented in Fig. 6. The increase of NOS activity in vessels from $B_1^{-/-}$ and $B_2^{-/-}$ probably is attributed to increase in activity of eNOS or nNOS, since experiments performed in absence of Ca^{2+} to determine iNOS activity (Ca^{2+}-independent) showed similar results among strains.

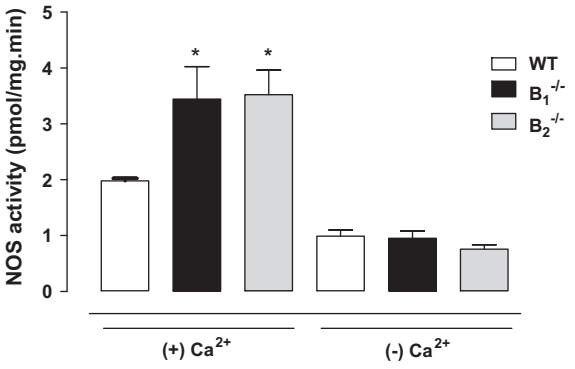

Fig. 6. Bars graph shows the NOS activity in presence or absence of Ca^{2+} to evaluate total NOS activity and inducible NOS activity, respectively, in homogenates of mesenteric vessels from WT, $B_1^{-/-}$ and $B_2^{-/-}$. Results are mean ± S.E.M., $n = 4$–5 for each group. $^*P < 0.05$ *vs* WT.

4. Discussion

The advent of potent and selective B_1 and B_2 receptor antagonists has permitted to assess the role of kinins in several biological systems; however, receptor antagonists are not devoid of unspecificity. The recent development of genetically engineered mice lacking the kinin B_1 and B_2 receptor has allowed the opportunity to investigate the physiological role of the kallikrein–kinin system in absence of pharmacological interventions. By analyzing the effect of vasoactive agents in mesenteric arterioles and measuring circulating and tissue NO production, we find several evidences that targeted deletion of kinin B_1 or B_2 receptor impairs endothelium-mediated vasodilation by reducing NO bioavailability.

Firstly, we observed that $B_2^{-/-}$ arterioles exhibit increase in basal perfusion pressure in comparison to WT and $B_1^{-/-}$. Although most of the studies have reported that $B_2^{-/-}$ are normotensive [1–3,11,12,26,35,37,39], these mice appear to exhibit exaggerated responses to hypertensive stimuli [3,11,12,15,20,21]. Thus, even without an essential role in blood pressure regulation, B_2 receptor is clearly related to modulation of vascular tonus and control of regional blood flow to the organs.

Considering that vasodilation induced by ACh is directly dependent on endothelial NO release [17] and that relaxating effect of SNP is attributed to direct NO delivery on the smooth muscle [8], our results demonstrate a severe impairment in the endothelial NO – dependent vasodilation in mesenteric arterioles from both $B_1^{-/-}$ and $B_2^{-/-}$. This finding is in agreement with previous data showing that the vasodepressor response to injection of ACh was shifted to the right in $B_2^{-/-}$ [2]. In the present study, we demonstrated for the first time that impaired vascular response to ACh is also present in the $B_1^{-/-}$ mice.

Contrasting in part with our results, a preserved response to ACh in $B_2^{-/-}$ mesenteric vessels has been previously related by Berthiaume et al. [6]. This discrepant result can be explained by marked differences in the methodology employed for vascular reactive experiments. Indeed, studies in mice mesenteric vessels have been performed under a wide range of flow velocities,

pre-contracting agents, Krebs composition and enzymatic blockers or other inhibitors added to the perfusion. In the present study, flow velocity was chosen on the basis of its ability to induce a sustained and sub-maximal vasoconstriction to NE (10 μmol/L), in the absence of other drugs. Vasodilator agents were tested in arterioles submitted to an approximate 80 mmHg increase in perfusion pressure, and, in this case, ACh (10 nmol) evoked a vasodilation around 12 mmHg, confirming that preparations were able to dilate.

In order to confirm whether a possible impairment in NO bioavailability in $B_1^{-/-}$ and $B_2^{-/-}$ could be responsible for the reduced ACh response, we analyzed plasmatic NO levels and vascular NO generation in both strains. As expected, we observed a significant reduction on circulating NO levels and basal NO release in mesenteric arterioles from $B_1^{-/-}$ and $B_2^{-/-}$. Similarly, studies have described lower nitrite/nitrate plasma levels [18] and reduced renal nitrite excretion [35] in $B_2^{-/-}$ when compared to WT mice. Moreover, induced hypertension by chronic NO synthesis inhibition is less pronounced in $B_2^{-/-}$ when compared to WT responses [20]. Therefore, B_2 receptor deletion may severely interfere with NO bioavailability. Our data show that, besides B_2, B_1 receptors are also involved in basal and stimulated NO metabolism.

Reduction in NO levels can occur through several potential mechanisms, such as reduced NOS enzymatic activity or increased NO inactivation [29]. Considering that the bioavailability of NO is largely dependent on NOS, we analyzed the NOS activity in mesenteric vessels by biochemical conversion of L-[3H] arginine to L-[3H] citrulline in presence of substrate and co-factors. Surprisingly, instead of the expected reduction, total NOS activity (Ca^{2+}-dependent) was elevated in homogenates of vessels from $B_1^{-/-}$ and $B_2^{-/-}$. These results are partially in agreement with Barbosa et al. [4], that observed a decrease in relaxating effect of SNP in stomach fundus from $B_1^{-/-}$, despite increase in iNOS activity and cGMP levels. These findings indicate that, at least in presence of supplementation with exogenous substrate and co-factors, NOS from both $B_1^{-/-}$ and $B_2^{-/-}$ is functional.

The present data do not give support for explaining the contrasting results about decreased NO levels accompanied by enhanced NOS activity in kinin knockout mice. One possible mechanism responsible for this could be the fact that uncoupling of NOS induces NOS-derived production of superoxide anion and hydrogen peroxide [14,36]. In this case, reduced NO bioavailability in $B_1^{-/-}$ and $B_2^{-/-}$ could be related to increase in vascular oxidative stress associated with elevated superoxide anion production and consequent NO inactivation. In fact, superoxide anion rapidly inactivates NO to form the highly reactive intermediate peroxynitrite, which represents a major potential pathway of NO reactivity and degradation [5,36]. Nevertheless, the generation of reactive oxygen species in $B_1^{-/-}$ and $B_2^{-/-}$ mice has not yet been consistently analyzed and further studies will be required to test this hypothesis.

5. Conclusion

In conclusion, the present study demonstrated that targeted deletion of B_1 or B_2 receptor gene in mice induces important alterations in the vascular reactivity of resistance vessels and NO metabolism. The severe impairment in the endothelial-mediated vasodilation accompanied by decreased NO bioavailability, despite the augmented NOS activity, strongly indicates an exacerbation of NO inactivation in $B_1^{-/-}$ and $B_2^{-/-}$. The present data provide valuable information in order to clarify the relevance of kinin receptors in regulating vascular physiology and may point to new approaches regarding its correlation with endothelial dysfunction, oxidative stress and NO availability.

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