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Antioxidant/Oxidant Status and Cardiac Function in Bradykinin B_1- and B_2-Receptor Null Mice

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Summary

Kinin-vasoactive peptides activate two G-protein-coupled receptors (R), B_1R (inducible) and B_2R (constitutive). Their complex role in cardiovascular diseases could be related to differential actions on oxidative stress. This study investigated impacts of B_1R or B_2R gene deletion in mice on the cardiac function and plasma antioxidant and oxidant status. Echocardiography-Doppler was performed in B_1R ($B_1R^{-/-}$) and B_2R ($B_2R^{-/-}$) deficient and wild type (WT) adult male mice. No functional alteration was observed in $B_2R^{-/-}$ hearts. $B_1R^{-/-}$ mice had significantly lowered fractional shortening and increased isovolumetric contraction time. The diastolic E and A waves velocity ratio was similar in all mice groups. Thus $B_1R^{-/-}$ mice provide a model of moderate systolic dysfunction, whereas $B_2R^{-/-}$ mice displayed a normal cardiac phenotype. Plasma antioxidant capacity (ORAC) was significantly decreased in both $B_1R^{-/-}$ and $B_2R^{-/-}$ mice whereas the vitamin C levels were decreased in $B_2R^{-/-}$ mice only. Plasma ascorbyl free radical was significantly higher in $B_1R^{-/-}$ compared to WT and $B_2R^{-/-}$ mice. Therefore, the oxidative stress index, ascorbyl free radical to vitamin C ratio, was increased in both $B_1R^{-/-}$ and $B_2R^{-/-}$ mice. Hence, B_1R and B_2R deficiency are associated with increased oxidative stress, but there is a differential imbalance between free radical production and antioxidant defense. The interrelationship between the differential B_1R and B_2R roles in oxidative stress and cardiovascular diseases remain to be investigated.

Key words

Doppler • Echocardiography • Kinin receptor • Knockout • Oxidative stress

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Introduction

Kinins are vasoactive peptides that play important roles in cardiovascular homeostasis and pathology (Regoli *et al.* 2012). They cause vasodilation, vascular permeability, and also inflammation. Kinin peptides, namely bradykinin (BK) and Lys-BK, are generated by cleavage of kininogens by plasma or tissue kallikreins. They are rapidly metabolized by kininase I and angiotensin 1-converting enzyme (Bhoola *et al.* 1992). Kinins and their C-terminal metabolites (des-Arg^9-BK and Lys-des-Arg^9-BK) activate G protein-coupled receptors (R), B_2R and B_1R, respectively (Regoli *et al.* 2001, Leeb-Lundberg *et al.* 2005). B_2R is constitutively expressed on endothelial cells and leads to prostacyclin and nitric oxide release through endothelial nitric oxide synthase. Kinins also induce a direct negative chronotropic effect mediated by the B_2R (Ribuot *et al.* 1993). B_1R is weakly expressed under physiological

conditions but plays a fundamental role in the cardiac function (Lauton-Santos *et al.* 2007). This receptor is strongly up-regulated by pro-inflammatory cytokines and oxygen free radicals (Couture and Girolami 2004). Oxidative stress, defined as imbalance between free radicals production and antioxidant defenses, is of great importance in the function of the kallikrein-kinin system (Dias *et al.* 2010, Kayashima *et al.* 2012). The main objective of this study was to investigate the impact of B_1R or B_2R deletion in mice on left ventricular (LV) function and plasma oxidative stress markers in physiological conditions.

Materials and Methods

Transgenic animals

$B_1R^{-/-}$ mice (n=9) on a 129/SvjxC57/J6 background (Pesquero *et al.* 2000) were obtained from Dr. Michael Bader's Laboratory (Max-Delbrück Center for Molecular Medicine, Berlin-Buch, Germany) and backcrossed 10 times to C57BL/6J as previously reported (Mori *et al.* 2008). $B_2R^{-/-}$ mice (n=9) on a C57BL/6J background were used (Blaes *et al.* 2012). Age-matched six-month-old male C57BL/6J wild type mice (WT, n=10) (Jackson Laboratories, Bar Harbor, Me) were used as control animals. The experimental study was approved by the local ethics committee and the investigators complied with authorization 21CAE057 from the French government, which agrees with the Directive 2010/63/EU of the European Parliament.

Echocardiographic analysis

Transthoracic micro-echocardiography was carried out as previously described (Delemasure *et al.* 2012) using a Vevo 770® (Visualsonics; Toronto, Canada) equipped with a 30-MHz high-frequency linear transducer. Briefly, cardiac parameters were measured in anesthetized mice (with isoflurane) in parasternal short and long axis views, and then the values were averaged. Heart rate was measured from the cardiac cycles using three consecutive beats. Left ventricular internal diameters and LV wall thickness determined by the inter-ventricular septum, posterior wall and anterior wall were measured at end diastole and systole. Systolic function was evaluated by the fractional shortening. LV mass was calculated.

Doppler analysis

Pulsed wave Doppler of the mitral valve was recorded from the apical four-chamber view in order to assess the diastolic function. Mitral Doppler flow spectra showed a higher early ventricular filling wave (E) and a lower late filling wave caused by atrial contraction (A). We measured the E and A wave velocities ratio (E/A), the isovolumetric relaxation time defined as the time interval between end of aortic outflow and onset of the mitral inflow, the isovolumetric contraction time defined as the time interval between end of mitral inflow and onset of the aortic outflow and informing on the dynamics of LV contraction.

Measurement of peroxyl radical scavenging activity in plasma

The antioxidant scavenging activity in plasma was evaluated by Oxygen Radical Absorbance Capacity (ORAC) miniaturized assay adapted from Cao *et al.* (1993). Fluorescence decay of allophycocyanin (APC, Sigma) due to a peroxyl radical generator (AAPH, 2,2-azobis(2-amidinopropane)-4-hydrochloride, Sigma) was measured over time using a Victor3V fluorimeter (PerkinElmer), with excitation and emission filters P620/8 and D665, respectively. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma), a soluble vitamin E analog, was used as antioxidant reference. ORAC was calculated using the net area under the decay curve and was expressed as µmol/l of trolox equivalent (TE). For each sample, the plasma antioxidant capacity was measured in quadruplicate.

Measurement of plasma vitamin C

After mixing the plasma with metaphosphoric acid (5 %), the concentrations of ascorbate and dehydroascorbic acid (total vitamin C) were measured by high performance liquid chromatography equipped with fluorescence detection (exc. = 360 nm; em. = 440 nm) (Tessier *et al.* 1996).

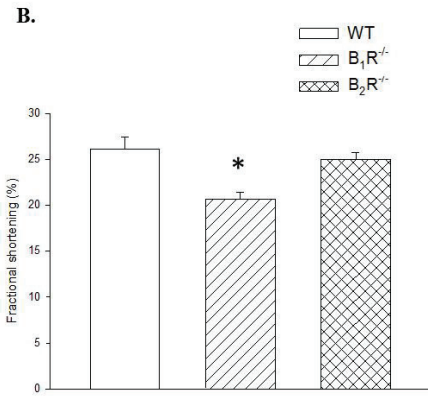
Detection of ascorbyl free radical in plasma and estimation of oxidative stress

Ascorbyl free radical was detected by Electron Spin Resonance (ESR) spectroscopy as previously reported (Vergely *et al.* 1998). Plasma was analyzed into a quartz capillary tube using a Bruker EMX X-band spectrometer. The formation of ascorbyl free radical was evaluated by the height of the signal and expressed in arbitrary units (AU). The plasma oxidative stress was estimated by the ascorbyl free radical to vitamin C ratio.

A.

	WT	$B_1R^{-/-}$	$B_2R^{-/-}$
Body weight (g)	29.9 ± 0.7	25.1 ± 0.3 *	28.4 ± 0.4
Heart rate (beats/min)	433 ± 9	441 ± 8	415 ± 6
LV anterior wall; diastole (mm)	0.77 ± 0.03	0.72 ± 0.01	0.71 ± 0.01
Inter ventricular septal wall; diastole (mm)	0.77 ± 0.03	0.72 ± 0.01	0.72 ± 0.02
LV internal diameter; diastole (mm)	4.44 ± 0.05	4.55 ± 0.05	4.45 ± 0.04
LV posterior wall; diastole (LVPW, mm)	0.77 ± 0.01	0.72 ± 0.01	0.77 ± 0.02
LV anterior wall; systole (mm)	1.01 ± 0.04	0.91 ± 0.02	0.91 ± 0.02
Inter ventricular septal wall; systole (mm)	1.02 ± 0.05	0.91 ± 0.03	0.91 ± 0.03
LV internal diameter; systole (mm)	3.29 ± 0.09	3.62 ± 0.07	3.34 ± 0.06
LV posterior wall; systole (mm)	1.07 ± 0.03	0.96 ± 0.02	1.03 ± 0.02
LV mass (mg)	132 ± 3	127 ± 2	127 ± 5
LV mass / body weight	4.4 ± 0.1	5.0 ± 0.1 *	4.5 ± 0.2

B.



C.

	WT	$B_1R^{-/-}$	$B_2R^{-/-}$
E- and A-waves velocities ratio	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
Isovolumetric relaxation time (ms)	18 ± 1	18 ± 1	18 ± 1
Ejection time (ms)	46 ± 3	46 ± 2	50 ± 2
Isovolumetric contraction time (ms)	21 ± 1	26 ± 2 *	20 ± 2

Fig. 1. Echocardiographic analysis of wild-type (WT) and B_1R or B_2R knockout mice ($B_1R^{-/-}$, $B_2R^{-/-}$). **A.** Measures concerning body weight, heart rate and left ventricle (LV). * $P<0.05$ versus WT. **B.** Analysis of fractional shortening in WT, B_1R or B_2R knockout mice. * $P<0.05$ versus WT. **C.** Doppler transmitral inflow in WT, $B_1R^{-/-}$ and $B_2R^{-/-}$ mice. * $P<0.05$ versus WT.

Statistical analysis

Data are expressed as mean ± SE. Differences between groups were evaluated by one-way ANOVA followed by pairwise comparisons by Student-Newman-Keuls test using SigmaPlot 11.0 (Systat software; San Jose, CA). A value of $P<0.05$ was considered statistically significant.

Results

Echocardiographic measurements of LV

The body weights were similar for $B_2R^{-/-}$ and WT mice but significantly lower for $B_1R^{-/-}$ mice (minus 13 %, P<0.05) (Fig. 1A). LV weights, measured by echocardiography, were similar for the three groups (mean of 128 mg). The heart to body weight ratio was thus significantly higher in $B_1R^{-/-}$ (5.0±0.1) compared to $B_2R^{-/-}$ (4.5±0.2) and WT (4.4±0.1) mice. The heart rates were stable and did not significantly differ between $B_1R^{-/-}$, $B_2R^{-/-}$ and WT mice, with a mean of 430 beats/min under anesthetized conditions (Fig. 1A). LV wall thickness measurements were similar for the knockout and WT mice. An upward trend, however not statistically significant, was noted for left ventricular internal diameter in $B_1R^{-/-}$ mice compared to $B_2R^{-/-}$ and WT mice. Consequently, $B_1R^{-/-}$ mice had a significant decline in fractional shortening compared to $B_2R^{-/-}$ and WT (–18 %, P<0.05) mice (Fig. 1B).

Doppler measurements of transmitral LV inflow

$B_1R^{-/-}$ and $B_2R^{-/-}$ mice had E and A wave velocities ratio and isovolumetric relaxation time similar to WT mice (Fig. 1C). The mitral ejection time was unchanged. An increased isovolumetric contraction time was observed in $B_1R^{-/-}$ compared to $B_2R^{-/-}$ and WT mice (+27 %, P<0.05).

Plasma Oxygen Radical Absorbance Capacity

The antioxidant status evaluated by ORAC was significantly lower in both $B_1R^{-/-}$ mice (1492±47 µmol/l TE) and $B_2R^{-/-}$ mice (1781±55 µmol/l TE) compared to WT mice (2322±84 µmol/l TE) (P<0.05) (Fig. 2A). $B_1R^{-/-}$ and $B_2R^{-/-}$ ORAC did not significantly differ.

Plasma vitamin C and ascorbyl free radical

Plasma total vitamin C levels did not significantly differ between $B_1R^{-/-}$ and WT mice (51±4 versus 60±4 µmol/l) (Fig. 2B). In contrast, it was significantly lower in $B_2R^{-/-}$ mice (36±5 µmol/l, P<0.05) compared to WT mice. The height of the signal intensity of the characteristic spectra of plasma ascorbyl free radical was similar in $B_2R^{-/-}$ and WT mice. In contrast, $B_1R^{-/-}$ mice exhibited a markedly higher signal intensity in comparison with WT or $B_2R^{-/-}$ mice (+41 %, P<0.05; Fig. 2C and 2D). The plasma oxidative stress, as evaluated by the ascorbyl free radical to vitamin C ratio, was increased in $B_1R^{-/-}$ and $B_2R^{-/-}$ mice as compared to WT mice (P<0.05). The difference between $B_1R^{-/-}$ and $B_2R^{-/-}$ mice did not reach statistical significance (Fig. 2E).

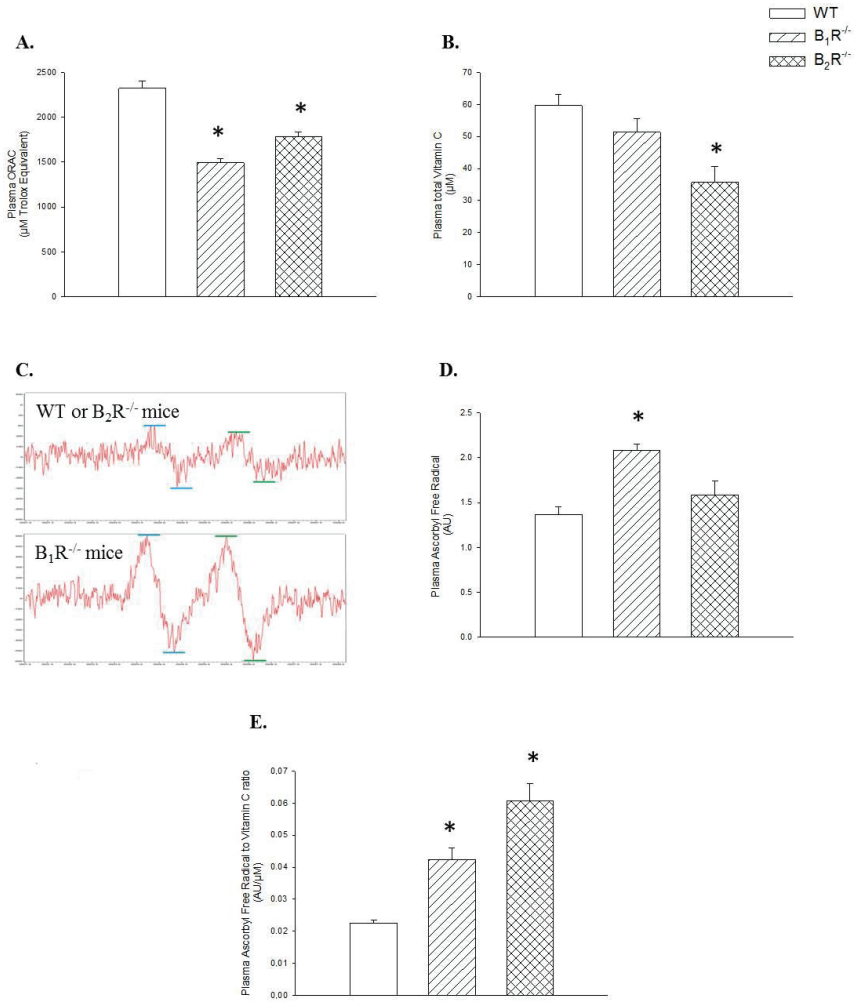


Fig. 2. Plasma antioxidant status in WT and $B_1R^{-/-}$, $B_2R^{-/-}$ mice. **A.** Determination using ORAC (Oxygen Radical Absorbance Capacity) measurements. **B.** Total plasma vitamin C quantified using HPLC. **P*<0.05 versus WT. **C.** Characteristic doublet of ascorbyl free radical ESR signal. ESR spectra were recorded with Bruker EMX X-band spectrometer using a quartz capillary tube at room temperature. **D.** Quantification of ascorbyl free radical determined by the measure of the ESR signal height. * *P*<0.05 versus WT. **E.** Plasma oxidative stress evaluated by the ascorbyl free radical to vitamin C ratio in WT and $B_1R^{-/-}$, $B_2R^{-/-}$ mice. **P*<0.05 versus WT.

Discussion

Antioxidant/oxidant status

Although we do not bring indications on myocardial oxidative stress markers, this study extends the knowledge of the phenotypes of B_1R and B_2R null mice. A decrease in plasma antioxidant scavenging activity (ORAC) was observed in both deficient strains. However, other plasma oxidative stress markers were differentially altered. Plasma total vitamin C concentration was decreased in $B_2R^{-/-}$ mice but not in $B_1R^{-/-}$ mice, suggesting that B_2R deficiency impacted synthesis and/or recycling of ascorbate. Total vitamin C (L-ascorbic acid and its oxidized form, dehydroascorbic acid) acts as essential water-soluble antioxidant in plasma (Farbstein *et al.* 2010). In humans, vitamin C must be provided in the diet while mice can synthesize ascorbate from glucose in the liver by the L-gulonolactone oxidase. Reduction of total vitamin C may result from increased renal excretion or to decreased liver synthesis. At physiological pH, L-ascorbic acid primarily exists as ascorbate anion which, in presence of oxygen free radicals, results in ascorbyl free radical, stable enough to be detected by ESR spectroscopy in plasma (Vergely *et al.* 1998). The plasma ascorbyl free radical content was only increased in $B_1R^{-/-}$. Nevertheless, the ascorbyl free radical to vitamin C ratio, index of oxidative stress (Courderot-Masuyer *et al.* 2000), was markedly increased in both $B_1R^{-/-}$ and $B_2R^{-/-}$ mice. The alterations of the antioxidant/oxidant status in $B_1R^{-/-}$ and $B_2R^{-/-}$ mice versus WT mice indicate that both kinin receptors control the oxidative balance under physiological conditions, although by different routes. In pathology, the B_1R and B_2R roles are distinct (Kayashima *et al.* 2012). B_1R is involved in insulin resistance and in early diabetes by increasing oxidative stress and pro-inflammatory mediators and later vascular alterations (Dias *et al.* 2012, Pouliot *et al.* 2012) while B_2R is involved in cardiovascular protection (Couture and Girolami 2004) and is necessary for cardioprotective effects provided by angiotensin-converting enzyme inhibitors, even if B_1R can contribute to protective effects (Duka *et al.* 2008).

Left ventricular systolic and diastolic functions

Previous evaluations of *in vivo* cardiac function in kinin receptor deficient mice brought somehow variable results under physiological conditions. No echocardiographic alteration was reported for 10-week $B_2R^{-/-}$ male mice (Yang *et al.* 2001, Duka *et al.* 2008) or 5-month female (Trabold *et al.* 2002) or in male mice of several $B_1R^{-/-}$ strains (Duka *et al.* 2008, Westermann *et al.* 2009, Wende *et al.* 2010). In contrast, diastolic LVID was increased in female 12-week-old $B_1R^{-/-}$ mice (Xu *et al.* 2009), and 2-month-old $B_2R^{-/-}$ mice showed higher LV mass and LVID at end-systole together with lower ejection fraction (Osorio *et al.* 2008). Older $B_2R^{-/-}$ mice (12-month-old) had moderately altered LV contractility and diastolic function (Madeddu *et al.* 1997). Alterations were more severe in the 129/J strain, showing the impact of interaction of B_2R signaling with other genetic determinant (two renin gene copy number) (Maestri *et al.* 2003). Collectively, described cardiac alterations of $B_1R^{-/-}$ and $B_2R^{-/-}$ mice remain moderate. Discrepancies may result from variation in the physiological conditions, i.e. gender, age or genetic background, isolated hearts or anesthetized animals or from differences in the methods to evaluate cardiac function. In this present study, a normal cardiac phenotype was observed in $B_2R^{-/-}$ mice. Although not significantly, most ventricular wall measures (posterior or anterior wall, in systole or diastole) were slightly lower for $B_1R^{-/-}$ hearts. These results argue for conditions of mild volume overload causing cavity dilation and thinning of the ventricular wall (Ram *et al.* 2011). Some measurements obtained from WT mice appeared lower than previously published values (Ram *et al.* 2011); this might be due to anesthesia-induced minor cardiac depression (also suggested by heart beats <500/min). However, in our study anesthesia conditions were comparable for the three mice strains. We showed a decrease of fractional shortening in $B_1R^{-/-}$ 6-month-old male mice. Consistently, previous *ex vivo* data showed a lower systolic function without hypertrophy in isolated hearts of 16-week-old male mice (Lauton-Santos *et al.* 2007). In B_1R or B_2R deficient mouse hearts, the other kinin receptor was found up-regulated in physiological state (Duka *et al.* 2008). Although moderate, the present systolic dysfunction in the $B_1R^{-/-}$ hearts may be the consequence of B_2R up-regulation and subsequent excessive negative chronotropic effect (Ribuot *et al.* 1993) but may also argued for a role of B_1R in the control of basal cardiac function. Our study is the first to assess the diastolic LV function by noninvasive conventional Doppler in mice lacking B_1R or B_2R. Transmitral pressure gradient and LV filling pressure were estimated as generally performed in patients. There was no difference between $B_1R^{-/-}$, $B_2R^{-/-}$ and WT mice with regard to the diastolic function, as evaluated by the early to late diastolic filling ratio and the time between aortic valve closure and mitral valve opening. A prolonged time between mitral valve closure and aortic valve opening was observed in $B_1R^{-/-}$ mice, which confirms an impaired LV contraction.

Conclusion

Deficiency in either kinin receptor altered the plasma oxidant/antioxidant balance, however with specific patterns. In addition, B_1R deficiency induced a moderate systolic dysfunction consistent with a role of B_1R in the control of the basal cardiac function. Consequences of such differential roles of B_1R and B_2R remain to be investigated in oxidative pathological conditions and metabolic cardiovascular diseases.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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