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## Protective role of AT<sub>2</sub> and B<sub>1</sub> receptors in kinin B<sub>2</sub>-receptor-knockout mice with myocardial infarction

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### Abstract

AT<sub>2</sub>Rs [AngII (angiotensin II) type 2 receptors] contribute to the cardioprotective effects of angiotensin II receptor blockers, possibly via kinins acting on the B<sub>1</sub>R (B<sub>1</sub> receptor) and B<sub>2</sub>R (B<sub>2</sub> receptor). Recent studies have shown that a lack of B<sub>2</sub>R up-regulates B<sub>1</sub>R and AT<sub>2</sub>R; however, the pathophysiological relevance of such an event remains unclear. We hypothesized that up-regulation of AT<sub>2</sub>R and B<sub>1</sub>R compensates for the loss of B<sub>2</sub>R. Blockade of AT<sub>2</sub>R and/or B<sub>1</sub>R worsens cardiac remodelling and dysfunction following MI (myocardial infarction) in B<sub>2</sub>R<sup>-/-</sup> (B<sub>2</sub>-receptor-knockout mice). B<sub>2</sub>R<sup>-/-</sup> mice and WT (wild-type) controls were subjected to sham MI or MI and treated for 4 weeks with (i) vehicle, (ii) a B<sub>1</sub>R-ant (B<sub>1</sub>R antagonist; 300 μg/kg of body weight per day), (iii) an AT<sub>2</sub>R-ant [AT<sub>2</sub> receptor antagonist (PD123319); 20 mg/kg of body weight per day], or (iv) B<sub>1</sub>R-ant + AT<sub>2</sub>R-ant. B<sub>2</sub>R<sup>-/-</sup> mice had a greater MCSA (myocyte cross-sectional area) and ICF (interstitial collagen fraction) at baseline and after MI compared with WT controls. Cardiac function and increase in macrophage infiltration, TGFβ<sub>1</sub> (transforming growth factor β<sub>1</sub>) expression and ERK1/2 (extracellular-signal-regulated kinase 1/2) phosphorylation post-MI were similar in both strains. Blockade of AT<sub>2</sub>R or B<sub>1</sub>R worsened cardiac remodelling, hypertrophy and dysfunction associated with increased inflammation and ERK1/2 phosphorylation and decreased NO excretion in B<sub>2</sub>R<sup>-/-</sup> mice, which were exacerbated by dual blockade of B<sub>1</sub>R and AT<sub>2</sub>R. No such effects were seen in WT mice. Our results suggest that, in the absence of B<sub>2</sub>R, both B<sub>1</sub>R and AT<sub>2</sub>R play important compensatory roles in preventing deterioration of cardiac function and remodelling post-MI possibly via suppression of inflammation, TGFβ<sub>1</sub> and ERK1/2 signalling.

### Keywords

angiotensin II type 2 receptor (AT<sub>2</sub> receptor); B<sub>1</sub> kinin receptor; B<sub>2</sub> kinin receptor; heart failure; inflammation; myocardial infarction; renin-angiotensin system

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**AUTHOR CONTRIBUTION** Jiang Xu performed major parts of the experiments, including surgeries on animals, echocardiography, histological staining, data analysis and preparation of the paper; Oscar A. Carretero participated in the experimental design, data interpretation and critical discussion of the paper; Liping Zhu performed mRNA experiments and data analysis; Edward Shesely was in charge of breeding and genotyping the mice; Nour-Eddine Rhaleb participated in the experimental design, data interpretation and discussion of the paper; Xiangguo Dai performed histological studies, including cutting and staining samples; Luchen Wang assisted Jiang Xu with the animal protocols and performed the Western blots; James Yang performed the statistical analyses; Xiao-Ping Yang oversaw the design and performance of the experiments, performed echocardiography, and edited and approved the final format of the paper.

## INTRODUCTION

CHF (chronic heart failure) due to myocardial ischaemia is a major cause of death in the U.S.A. and worldwide. Activation of the RAS (renin–angiotensin system) and release of AngII (angiotensin II) acting on the AT<sub>1</sub>Rs (AngII type 1 receptors) both play a crucial role in the pathophysiology of CHF. Blockade of AT<sub>1</sub>R with ARBs (angiotensin receptor blockers) has been shown to improve LV (left ventricular) function, regress LV remodelling and prolong survival in patients with CHF [1,2]. We and others [3–5] have demonstrated that the cardioprotective effects of ARBs are mediated in part via activation of AT<sub>2</sub>Rs (AngII type 2 receptors) due to increased circulating AngII during AT<sub>1</sub>R blockade. AngII in turn acts on AT<sub>2</sub>R and affords cardioprotection via a kinin-dependent pathway [6,7].

Two types of kinin receptors have been identified: B<sub>1</sub>R (B<sub>1</sub> receptor) and B<sub>2</sub>R. Most known cardiovascular actions of kinins are generally attributed to the constitutively expressed B<sub>2</sub>R, whereas the role of B<sub>1</sub>R is not well defined. Although B<sub>1</sub>R is weakly expressed under physiological conditions, it is markedly increased in response to pathological stimuli such as myocardial ischaemia [8,9]. We have reported previously that both B<sub>1</sub>R and B<sub>2</sub>R contribute to the cardioprotective effects of ARBs [3,8,10]. Up-regulation of the B<sub>1</sub>R is also demonstrated when the B<sub>2</sub>R is absent [11], which may compensate for the loss of B<sub>2</sub>R. Indeed, we have shown that blockade or deletion of B<sub>2</sub>R alone does not have a significant impact on cardiac morphology and function either under basal conditions or in animals with MI (myocardial infarction); however, blocking both B<sub>2</sub>R and B<sub>1</sub>R exacerbates cardiac remodelling and dysfunction post-MI [8]. Kakoki et al. [12] have also shown that renal ischaemia/reperfusion injury becomes more severe in mice lacking both B<sub>1</sub>R and B<sub>2</sub>R compared with a lack of B<sub>2</sub>R alone. Taken together, these studies suggest that up-regulation of B<sub>1</sub>R during deletion or blockade of B<sub>2</sub>R may represent a compensatory mechanism.

A lack of the B<sub>2</sub>R also reportedly up-regulates the AT<sub>2</sub>R. Shariat-Mader et al. [13] showed that, in B<sub>2</sub>R<sup>-/-</sup> mice, AT<sub>2</sub>R expression increased 8-fold in association with increased B<sub>1</sub>R. However, the functional or pathophysiological relevance of B<sub>1</sub>R and AT<sub>2</sub>R up-regulation in B<sub>2</sub>R<sup>-/-</sup> mice is not well understood. The present study is designed to test the hypothesis that, in the absence of B<sub>2</sub>R, AT<sub>2</sub>R and B<sub>1</sub>R are up-regulated, which compensates for the loss of B<sub>2</sub>R. Using B<sub>2</sub>R<sup>-/-</sup> mice subjected to MI, we studied whether blockade of AT<sub>2</sub>R and/or B<sub>1</sub>R in the absence of B<sub>2</sub>R increases inflammation and aggravates cardiac hypertrophy, fibrosis, dilatation and dysfunction. The roles of TGFβ<sub>1</sub> (transforming growth factor β<sub>1</sub>) and ERK (extracellular-signal-regulated kinase)1/2 signalling were also investigated.

## MATERIALS AND METHODS

### Animals

Male B<sub>2</sub>R<sup>-/-</sup> mice (B6; 129S7-bdkrb2<sup>tm1Jfh</sup>/J; stock number 002641) and WT (wild-type) controls (B6129SF2/J; stock number 101045) were purchased from Jackson Laboratories. Both strains were on a mixed genetic background of C57BL/6J and 129S7/SvEv. Animals were housed in an air-conditioned room with a 12 h light/12 h dark cycle, received standard chow and drank tap water. This study was approved by the IACUC (Institutional Animal Care and Use Committee) of the Henry Ford Health System. All studies were conducted in accordance with the NIH (National Institutes of Health) Guidelines for the Care and Use of Laboratory Animals.

### Induction of MI and experimental protocols

Mice 8–10 weeks of age were anaesthetized with sodium pentobarbital (50 mg/kg of body weight, intraperitoneal). An analgesic, buprenorphine (2.5 mg/kg of body weight, subcutaneous), was given 1 h before the operation and 6 h afterward. MI was induced by

ligating the coronary artery [3,14]. Briefly, a left thoracotomy was performed via the fourth intercostal space, the heart exposed and the pericardium opened. The left anterior descending coronary artery was ligated with an 8-0 silk suture. Acute myocardial ischaemia was deemed successful when the anterior wall of the LV became cyanotic and the ECG showed obvious ST segment elevation. The lungs were then inflated by increasing positive end-expiratory pressure and the thoracotomy site closed in layers. Mice were kept on a heating pad until they recovered. After the MI or sham MI, each strain was divided into (i) sham MI ( $n=13$  WT,  $n=12$  B<sub>2</sub>R<sup>-/-</sup>); (ii) MI+vehicle (saline) ( $n=9$  WT,  $n=9$  B<sub>2</sub>R<sup>-/-</sup>); (iii) MI +AT<sub>2</sub> receptor antagonist (AT<sub>2</sub>R-ant, PD123319; 20 mg/kg of body weight per day) ( $n=10$  WT,  $n=9$  B<sub>2</sub>R<sup>-/-</sup>); (iv) MI+B<sub>1</sub> receptor antagonist (B<sub>1</sub>R-ant, R892, 300  $\mu$ g/kg of body weight per day) ( $n=7$  WT,  $n=7$  B<sub>2</sub>R<sup>-/-</sup>); and (v) MI+B<sub>1</sub>R-ant+AT<sub>2</sub>R-ant ( $n=8$  WT,  $n=9$  B<sub>2</sub>R<sup>-/-</sup>). Doses of the antagonists were based on previously published work of ourselves and others [10,15–18]. All drugs were infused via an osmotic mini-pump intraperitoneally and started on the same day as coronary ligation and continued for 4 weeks.

### Blood pressure and cardiac function and remodelling

SBP (systolic blood pressure) and HR (heart rate) were measured in conscious mice using a non-invasive computerized tail-cuff system (BP-2000; Visitech) [19]. LV dimension and function were evaluated in awake mice using a Doppler echocardiograph equipped with a 15-MHz linear transducer (Acuson c256) as described previously [19,20].

### Histopathological study

At 4 weeks after MI, the heart was stopped at diastole by intraventricular injection of 15% KCl (50  $\mu$ l). The heart, lungs and liver were weighed, corrected by BW (body weight) and expressed as mg/10 g of BW to assess LV hypertrophy and lung congestion. The LV was sectioned transversely into three slices for assessment of infarct size, MCSA (myocyte cross-sectional area) and ICF (interstitial collagen fraction) as previously described [19]. Images were taken at  $\times 400$  magnification and analysed with a MicroSuite computerized image analysis system (Olympus).

### Immunohistochemical staining for macrophages

Frozen sections of the left ventricle (6  $\mu$ m) were stained with a rat anti-(mouse CD68) monoclonal antibody (1:200 dilution; AbD Serotec), a marker for macrophages, as described previously [19,21]. CD68-positive cells (reddish/brown staining) were recognized as macrophages and are expressed as number of cells per mm<sup>2</sup> of myocardium.

### ERK1/2 phosphorylation and TGF $\beta$ <sub>1</sub> protein expression

LV tissue was homogenized in lysis buffer with protease inhibitors. pERK1/2 (phosphor-ERK1/2) and TGF $\beta$ <sub>1</sub> protein expression was analysed by Western blot [19,22]. pERK1/2 was normalized by total ERK1/2 and TGF $\beta$ <sub>1</sub> proteins by GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Results were expressed as the ratio of the density of specific bands to the corresponding internal controls.

### Urinary NO and creatinine

Urine was collected from the bladder under anaesthesia 4 weeks after MI to measure NO<sub>x</sub> (combined nitrate + nitrite), which are stable end products of NO [23]. NO<sub>x</sub> was measured with a colorimetric assay kit (Cayman Chemical). Urinary creatinine was measured using a commercial kit (Sigma). Urinary NO<sub>x</sub> excretion was corrected by creatinine and expressed as the ratio of NO<sub>x</sub> to creatinine.

## Real-time PCR of B<sub>1</sub>R, B<sub>1</sub>R<sub>2</sub> and AT<sub>2</sub>R mRNA expression

Additional sham-operated mice (six WT and six B<sub>2</sub>R<sup>-/-</sup>) and mice with MI (seven WT and five B<sub>2</sub>R<sup>-/-</sup>) were used to determine B<sub>1</sub>R, B<sub>1</sub>R<sub>2</sub> and AT<sub>2</sub>R mRNA expression. Total RNA was isolated from mouse hearts using an RNeasy fibrous tissue kit (Qiagen). TaqMan gene expression assays from Applied Biosystems were used to assess expression of B<sub>1</sub>R (Mm04207315), B<sub>2</sub>R (Mm00437788), AT<sub>2</sub>R (Mm01341373) and GAPDH (Mm99999915). Real-time PCR conditions were: one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Gene expression was quantified and analysed using the comparative C<sub>t</sub> (cycle threshold value) method as described in the Applied Biosystems user bulletin. All values were corrected to GAPDH as an internal control and the value in sham-operated WT mice was set to a value of 1.0. Changes in mRNA expression after MI were expressed as 2<sup>-ΔΔC<sub>t</sub></sup>.

## Data analysis

All results are expressed as means ± S.E.M. A two-sample Student's *t* test was used to compare differences between strains or between treatments within strains. When multiple comparisons were performed, Hochberg's step-up procedure was used to adjust *P* values. The family-wise type I error rate was set at 0.05.

## RESULTS

### SBP, HR, BW and tissue weight and infarct size

SBP, HR and BW were similar among groups with or without MI. In the sham-operated mice, LV and total heart weight were heavier in B<sub>2</sub>R<sup>-/-</sup> than in WT and MI increased heart weight the same in both strains. Lung weight was similar among groups with or without MI in either strain. However, liver weight tended to be higher in all B<sub>2</sub>R<sup>-/-</sup> groups, although the increase reached statistical significance only in mice treated with B<sub>1</sub>R-ant with or without AT<sub>2</sub>R-ant. Infarct size was similar between strains with or without treatment (Table 1).

### Cardiac function and remodelling

Knockout of B<sub>2</sub>R had no effect on basal LV chamber size and function. MI increased LVDD (LV diastolic dimension) and decreased EF (ejection fraction) similarly in both strains. Blocking AT<sub>2</sub>R or B<sub>1</sub>R further increased LVDD and decreased EF in B<sub>2</sub>R<sup>-/-</sup> mice, and these changes were exaggerated when both AT<sub>2</sub>R and B<sub>1</sub>R were blocked. None of these effects were seen in the WT mice (Figure 1).

### Myocyte hypertrophy and cardiac interstitial fibrosis

Under basal conditions (sham MI), B<sub>2</sub>R<sup>-/-</sup> mice exhibited increased MCSA and ICF compared with WT. MI increased MCSA and ICF in both strains and this increase was greater in B<sub>2</sub>R<sup>-/-</sup> mice. AT<sub>2</sub>R-ant, B<sub>1</sub>R-ant or the combination of these two had no significant effect on MCSA and ICF in WT. However, in B<sub>2</sub>R<sup>-/-</sup> mice both AT<sub>2</sub>R-ant and B<sub>1</sub>R-ant alone increased MCSA and ICF further and these responses were exacerbated by dual blockade of AT<sub>2</sub>R and B<sub>1</sub>R (Figure 2).

### Macrophage infiltration, TGFβ<sub>1</sub> protein expression and ERK1/2 phosphorylation in the heart

A few macrophages were seen infiltrating the myocardium in both WT and B<sub>2</sub>R<sup>-/-</sup> mice with sham MI. Macrophage infiltration was increased after MI and no strain difference was noticed. Blocking either AT<sub>2</sub>R or B<sub>1</sub>R increased the number of infiltrated macrophages only in B<sub>2</sub>R<sup>-/-</sup> mice. Dual blockade of AT<sub>2</sub>R and B<sub>1</sub>R markedly elevated macrophage infiltration, which was also only seen in B<sub>2</sub>R<sup>-/-</sup> (Figure 3). Cardiac TGFβ<sub>1</sub> protein

expression did not differ between sham-operated WT and  $B_2R^{-/-}$  animals and rose similarly in response to MI in both strains.  $AT_2R$ -ant or  $B_1R$ -ant significantly enhanced TGF- $\beta_1$  protein expression in  $B_2R^{-/-}$  mice but not in WT mice, whereas dual blockade of  $AT_2R$  and  $B_1R$  markedly enhanced TGF- $\beta_1$  expression in both strains, but the response was greater in  $B_2R^{-/-}$  mice (Figure 4). ERK1/2 phosphorylation was similar between sham-operated groups and increased similarly after MI in both strains.  $AT_2R$ -ant or  $B_1R$ -ant significantly increased ERK1/2 phosphorylation further only in  $B_2R^{-/-}$  mice, while blocking both  $AT_2R$  and  $B_1R$  enhanced this effect in  $B_2R^{-/-}$  but not in WT mice (Figure 5).

### Urinary $NO_x$

Urinary  $NO_x$  concentration was not different in sham-operated or vehicle-treated groups between strains.  $AT_2R$ -ant and  $B_1R$ -ant significantly decreased urinary  $NO_x$  only in  $B_2R^{-/-}$  mice. Dual inhibition of  $AT_2R$  and  $B_1R$  only lowered urinary  $NO_x$  further in  $B_2R^{-/-}$  mice (Figure 6).

### Cardiac $B_1R$ , $B_2R$ and $AT_2R$ mRNA expression

Basal  $B_1R$  and  $AT_2R$  mRNA expression were significantly higher in  $B_2R^{-/-}$  mice compared with WT.  $B_2R$  mRNA was undetectable in  $B_2R^{-/-}$  mice. MI increased  $B_1R$ ,  $B_2R$  and  $AT_2R$  mRNA expression in WT mice, whereas in  $B_2R^{-/-}$  mice expression of  $B_1R$  and  $AT_2R$  tended to be higher in response to MI, although these changes did not reach statistical significance (Figure 7).

## DISCUSSION

In the present study, we demonstrated that deletion of  $B_2R$  resulted in up-regulation of  $B_1R$  and  $AT_2R$  without altering SBP or cardiac function, although myocyte size and collagen deposition were slightly but significantly increased under basal conditions. Likewise  $B_2R$  deletion had no significant impact on MI-induced cardiac hypertrophy, fibrosis or dysfunction compared with WT mice. These findings may suggest that up-regulation of  $B_1R$  and  $AT_2R$  in  $B_2R^{-/-}$  mice may have compensated for the loss of  $B_2R$ , maintaining cardiac and haemodynamic homeostasis and preventing deterioration of cardiac dysfunction and remodelling post-MI. This hypothesis is supported by our observation that blocking either  $AT_2R$  or  $B_1R$  exacerbated cardiac hypertrophy and dysfunction in  $B_2R^{-/-}$  mice, which was worsened by concomitant blockade of  $AT_2R$  and  $B_1R$ . Moreover, these detrimental cardiac effects were associated with increased macrophage infiltration, cardiac TGF $\beta_1$  protein expression and ERK1/2 phosphorylation as well as decreased urinary  $NO_x$  excretion. Together these findings suggest that up-regulation of  $AT_2R$  and  $B_1R$  plays an important cardioprotective role in compensating for the loss of  $B_2R$ , particularly in mice with myocardial ischaemia. Suppressing this compensation promotes exacerbation of MI-induced cardiac hypertrophy, fibrosis and dysfunction, possibly via an enhanced inflammatory response and ERK1/2 activation.

The effects of  $B_2R$  deletion on regulation of SBP and cardiac structure and function remain in question. We and others reported that lack of  $B_2R$  ( $B_2R^{-/-}$  mice on a C57BL/6J background) did not alter either SBP or cardiac phenotype under basal conditions, nor did it affect cardiac remodelling and function post-MI or in response to DOCA (deoxycorticosterone acetate)-salt-induced hypertension, although the therapeutic effects of ACEi (angiotensin-converting enzyme inhibitor) and ARB on cardiac remodelling and dysfunction were diminished [3,24,25]. In contrast, others have reported that lack of  $B_2R$  ( $B_2R^{-/-}$  mice on a 129/J background) increased SBP and caused cardiac hypertrophy and dilated cardiomyopathy by 40 weeks of age [11,26,27]. Thus the discrepancy observed in  $B_2R^{-/-}$  mice might be attributed to differences in genetic background, age and/or the use of

WT controls. In the present study, we compared  $B_2R^{-/-}$  mice on a mixed background of C57BL/6J and 129S7/SvEv with B6129SF2/J mice (WT) and found that the  $B_2R^{-/-}$  tended to have a higher SBP associated with a greater heart weight, MCSA and ICF under basal conditions, suggesting that  $B_2R$  may be involved in regulation of BP and cardiac homeostasis, which may be largely dependent on their genetic background.

Recent studies suggested that deletion of  $B_2R$  up-regulates  $B_1R$  and/or  $AT_2R$  [11,13,28,29], which is confirmed by our current observation that both  $B_1R$  and  $AT_2R$  mRNA expression were increased in  $B_2R^{-/-}$  mice. However, the functional significance of  $B_1R$  and/or  $AT_2R$  up-regulation is not fully understood. In our study, a lack of  $B_2R$  did not have a significant impact on cardiac remodelling and dysfunction post-MI, which may be partially due to up-regulation of  $B_1R$  and  $AT_2R$  that may assume some effects of  $B_2R$ . However, blocking  $B_1R$  and/or  $AT_2R$  in  $B_2R^{-/-}$  mice worsened LV chamber dilatation and cardiac hypertrophy, fibrosis and dysfunction, supporting our hypothesis that up-regulation of  $B_1$  and  $AT_2$  may compensate for loss of  $B_2R$ .

Whether the role of  $B_1R$  expression in the cardiovascular and renal system is beneficial or harmful remains inconclusive. We and others have shown that ACEi and ARB improved cardiac function and remodelling post-MI associated with up-regulation of  $B_1R$  expression and these effects were diminished in  $B_1R^{-/-}$  mice or in rats treated with a  $B_1R$  antagonist, although deletion or inhibition of  $B_1R$  did not have a significant impact on cardiac remodelling and dysfunction post-MI [30,31]. These findings may suggest that the cardioprotective actions of ACEi or ARB require  $B_1R$ . Previously, Schulze-Topphoff et al. [32] reported that in a mouse model of EAE (experimental autoimmune encephalomyelitis), activation of  $B_1R$  with the agonist, Sar-[D-Phe]des-Arg<sup>9</sup>-BK, reduced T-lymphocyte infiltration into the brain and improved the symptoms of EAE, whereas inhibition or deletion of  $B_1R$  enhanced the immune response and worsened severity of the disease [32]. Merino et al. [33] reported that  $B_1R$  deficiency aggravated atherosclerosis and promoted aneurysm formation in ApoE<sup>-/-</sup> (apolipoprotein E-knockout) mice [33]. All together, these reports support the concept that  $B_1R$  plays a cardioprotective, anti-inflammatory and anti-atherogenic role. On the other hand, contradictory results have also been reported, showing that blocking or deleting  $B_1R$  protects the heart against injury and myocyte death [34,35]. At present, we have no good explanation for these discrepancies.

It has been suggested that activation of ERK1/2 promotes cardiac inflammation, hypertrophy and fibrosis [36]. Kinins acting on  $B_1R$  and/or  $B_2R$  reduced inflammation by inhibiting MAPK (mitogen-activated protein kinase) activation, including ERK1/2 [37,38], whereas blocking  $B_1R$  and/or  $B_2R$  increases ERK1/2 phosphorylation and promotes cardiac inflammation and remodelling. Activation of  $AT_2R$  also reportedly attenuated TGF $\beta_1$ -induced ERK activation [39]. In the present study, we found that dual blockade of  $B_1R$  and  $AT_2R$  enhanced ERK1/2 phosphorylation and inflammatory responses as indicated by increased macrophage infiltration and TGF $\beta_1$  protein expression, suggesting that enhanced ERK1/2 phosphorylation and inflammation may contribute to the deterioration of cardiac remodelling and dysfunction observed in  $B_2R^{-/-}$  mice treated with both  $B_1R$  and  $AT_2R$  antagonists.

Studies have suggested that activation of eNOS (endothelial NO synthase) and enhanced NO production may be important mediators involved in signalling mechanisms responsible for  $B_1R$ -,  $B_2R$ - and/or  $AT_2R$ -induced cardioprotection [40,41]. We previously reported that overexpression and activation of  $AT_2R$  in cultured ECs (endothelial cells) stimulated release of NO and this stimulation was diminished by  $B_2R$  blockade [42], suggesting that the kinin/NO pathway plays an important role in mediating the cardioprotective effects of  $AT_2R$ . It has been suggested that NO inhibits TGF $\beta_1$ , thus suppressing ERK1/2 phosphorylation,

reducing proliferation, inflammation, oxidative stress and improving cardiac remodelling and dysfunction [43–45]. In the present study, we found that the levels of urinary NO in B<sub>2</sub>R<sup>-/-</sup> mice were significantly lower than in WT post-MI when treated with AT<sub>2</sub>R-ant and/or B<sub>1</sub>R-ant. It is possible that reduced NO production due to blockade of AT<sub>2</sub>R and B<sub>1</sub>R in B<sub>2</sub>R<sup>-/-</sup> enhances TGFβ<sub>1</sub> expression and ERK1/2 signalling, which promotes inflammation and cardiac hypertrophy, fibrosis and dysfunction post-MI.

### Limitations of the study

The present study has demonstrated that up-regulation of AT<sub>2</sub>R and B<sub>1</sub>R in B<sub>2</sub>R<sup>-/-</sup> mice may compensate for loss of B<sub>2</sub>R and play an important role in the cardioprotective effect of ARB. Our end points focused on functional and pathophysiological and histological remodelling of the heart; thus the detailed molecular and cellular mechanisms and signalling pathways that lead to NO synthase phosphorylation and TGFβ<sub>1</sub> and ERK activation were not emphasized, which will be our future focus and tested in both *in vivo* and *in vitro* studies.

In summary, we have shown that deletion of B<sub>2</sub>R unregulated cardiac AT<sub>2</sub>R and B<sub>1</sub>R expression, which may compensate for loss of the beneficial effects of B<sub>2</sub>R on remodelling and dysfunction in mice with MI, since blocking both AT<sub>2</sub>R and B<sub>1</sub>R worsened cardiac remodelling, hypertrophy and dysfunction in association with enhanced inflammatory responses and ERK1/2 phosphorylation and decreased urinary NO excretion. Thus AT<sub>2</sub>R, B<sub>1</sub>R and B<sub>2</sub>R may all play an important role in protecting the heart from MI-induced damage by increasing NO production and suppressing inflammatory cell infiltration, TGFβ<sub>1</sub> protein expression and ERK1/2 phosphorylation.

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### Abbreviations

<b>ACEi</b>	angiotensin-converting enzyme inhibitor
<b>AngII</b>	angiotensin II
<b>ARB</b>	angiotensin receptor blocker
<b>AT<sub>1</sub>R</b>	AngII type 1 receptor
<b>AT<sub>2</sub>R</b>	AngII type 2 receptor
<b>AT<sub>2</sub>R-ant</b>	AT <sub>2</sub> R antagonist
<b>B<sub>1</sub>R</b>	B <sub>1</sub> receptor
<b>B<sub>1</sub>R-ant</b>	B <sub>1</sub> R antagonist
<b>B<sub>2</sub>R</b>	B <sub>2</sub> receptor
<b>BW</b>	body weight
<b>CHF</b>	chronic heart failure
<b>CVD</b>	cardiovascular disease
<b>EAE</b>	experimental autoimmune encephalomyelitis
<b>EF</b>	ejection fraction

<b>ERK</b>	extracellular-signal-regulated kinase
<b>GAPDH</b>	glyceraldehyde-3-phosphate dehydrogenase
<b>HR</b>	heart rate
<b>ICF</b>	interstitial collagen fraction
<b>LV</b>	left ventricular
<b>LVDd</b>	LV diastolic dimension
<b>MCSA</b>	myocyte cross-sectional area
<b>MI</b>	myocardial infarction
<b>NO<sub>x</sub></b>	combined nitrate + nitrite
<b>pERK1/2</b>	phospho-ERK1/2
<b>TGFβ<sub>1</sub></b>	transforming growth factor β <sub>1</sub>
<b>RAS</b>	renin–angiotensin system
<b>SBP</b>	systolic blood pressure
<b>WT</b>	wild-type

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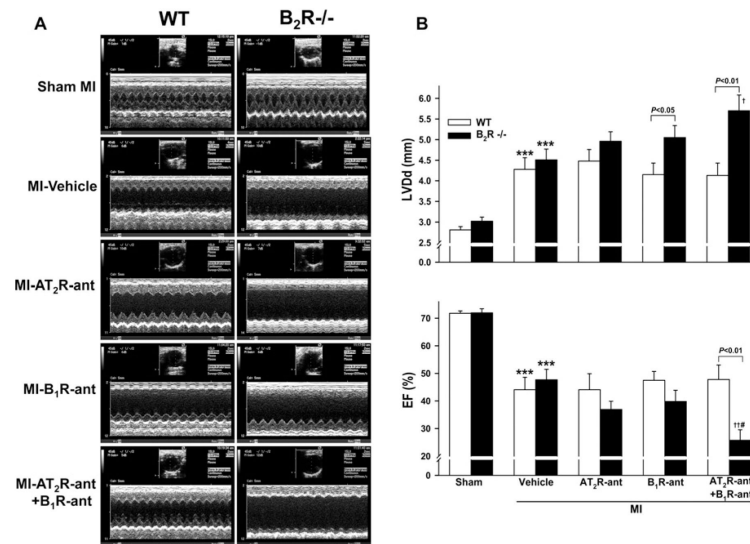


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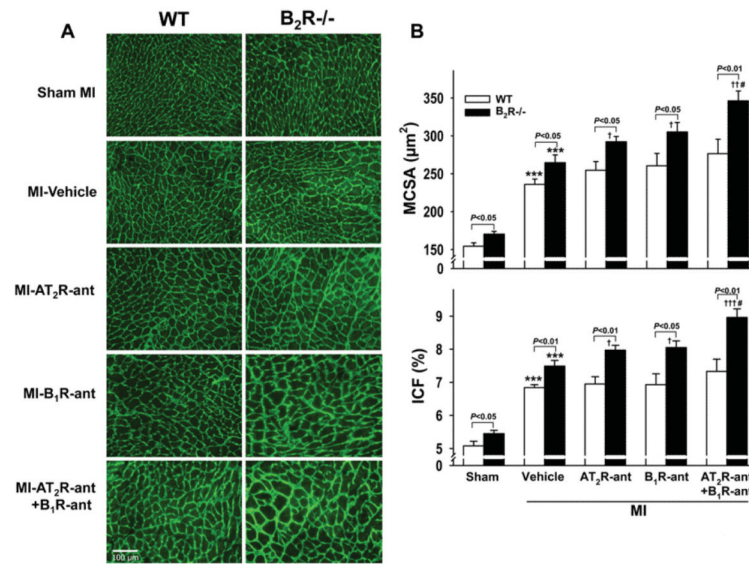
### CLINICAL PERSPECTIVES

- CVD is the leading cause of death in the U.S.A. and world-wide. Activation of the RAS plays a crucial role in the pathophysiology of CVD and up until now the standard therapy has been inhibition of the RAS using ACEi and/or ARB.
- We and others have demonstrated that activation of B<sub>1</sub>R, B<sub>2</sub>R and/or AT<sub>2</sub>R plays an important role in the cardioprotective effects of ACEi and ARBs. The present study helps to clarify the interaction between B<sub>2</sub>R, B<sub>1</sub>R and AT<sub>2</sub>R and the mechanisms by which these receptors offer cardioprotection.
- From a clinical perspective, understanding these receptor-mediated mechanisms may help us to develop new therapeutic strategies to treat CVD, such as targeted activation of AT<sub>2</sub>R, B<sub>2</sub>R and/or B<sub>1</sub>R, or combining them with an ACEi or ARB.



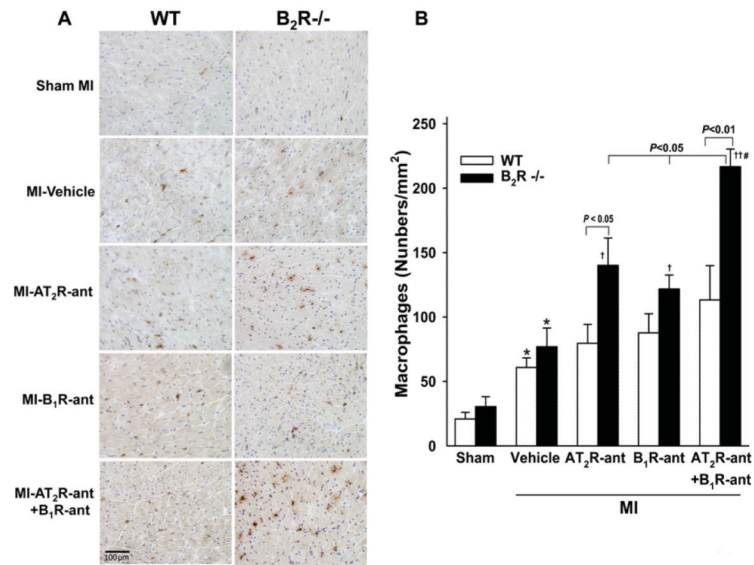
**Figure 1. Effect of AT<sub>2</sub>R and/or B<sub>1</sub>R blockade on LV EF and LVDD in WT and kinin B<sub>2</sub>R<sup>-/-</sup> mice post-MI**

(A) Representative two-dimensional M-mode ECGs of conscious WT and B<sub>2</sub>R<sup>-/-</sup> mice with sham MI or MI treated with vehicle, the AT<sub>2</sub>R antagonist PD123319 (AT<sub>2</sub>R-ant), the kinin B<sub>1</sub> receptor antagonist R892 (B<sub>1</sub>R-ant) or AT<sub>2</sub>R-ant plus B<sub>1</sub>R-ant for 4 weeks. (B) Quantitative analysis of LVDD (top) and EF (bottom). \*\*\* $P < 0.001$  compared with sham within strain; † $P < 0.05$  and †† $P < 0.01$  compared with vehicle within strain; # $P < 0.05$  compared with AT<sub>2</sub>R-ant or B<sub>1</sub>R-ant alone within strain.



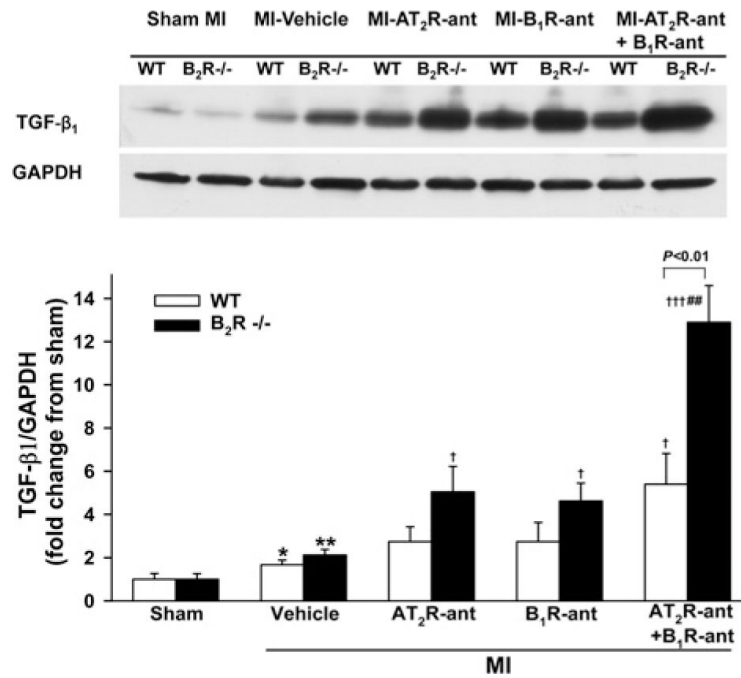
**Figure 2. Effect of AT<sub>2</sub>R and/or B<sub>1</sub>R blockade on MCSA and ICF in WT and B<sub>2</sub>R<sup>-/-</sup> mice post-MI**

(A) Representative images showing MCSA (delineated by the interstitial space) and interstitial collagen deposition (green staining) in WT and B<sub>2</sub>R<sup>-/-</sup> given vehicle, AT<sub>2</sub>R-ant, B<sub>1</sub>R-ant or AT<sub>2</sub>R-ant + B<sub>1</sub>R-ant. (B) Quantitative analysis of MCSA (top) and ICF (bottom). \*\*\* $P < 0.001$  compared with sham within strain; † $P < 0.05$  and ††† $P < 0.001$  compared with vehicle within strain; # $P < 0.05$  compared with AT<sub>2</sub>R-ant or B<sub>1</sub>R-ant alone within strain.



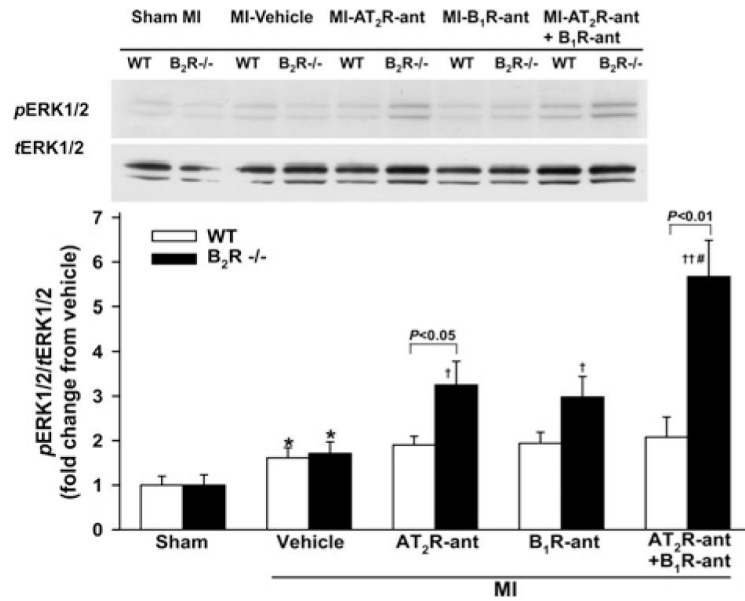
**Figure 3. Effect of AT<sub>2</sub>R and/or B<sub>1</sub>R blockade on macrophage infiltration in the LV myocardium in WT and B<sub>2</sub>R<sup>-/-</sup> post-MI**

(A) Representative images showing infiltrating macrophages (reddish-brown stained) in WT and B<sub>2</sub>R<sup>-/-</sup> given vehicle, AT<sub>2</sub>R-ant, B<sub>1</sub>R-ant or AT<sub>2</sub>R-ant + B<sub>1</sub>R-ant. (B) Quantification of the number of macrophages infiltrating the LV myocardium. \**P* < 0.05 compared with sham within strain; †*P* < 0.05 and ††*P* < 0.01 compared with vehicle within strain; #*P* < 0.05 compared with AT<sub>2</sub>R-ant or B<sub>1</sub>R-ant alone within strain.



**Figure 4. Effect of AT<sub>2</sub>R and/or B<sub>1</sub>R blockade on TGFβ<sub>1</sub> protein expression in WT and B<sub>2</sub>R<sup>-/-</sup> mice post-MI**

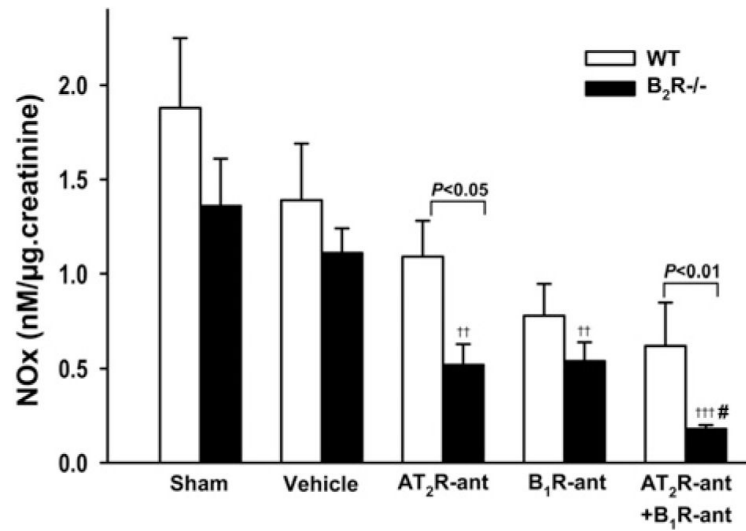
(A) Representative Western blots of TGFβ<sub>1</sub> and GAPDH. (B) Semi-quantitative analysis of TGFβ<sub>1</sub> protein corrected by GAPDH and expressed as fold change relative to sham controls within strain. \**P* < 0.05 and \*\**P* < 0.01 compared with sham within strain; †*P* < 0.05 and †††*P* < 0.001 compared with vehicle within strain; ##*P* < 0.01 compared with AT<sub>2</sub>R-ant or B<sub>1</sub>R-ant alone within strain.



**Figure 5. Effect of AT<sub>2</sub>R and/or B<sub>1</sub>R blockade on ERK1/2 phosphorylation in WT and B<sub>2</sub>R<sup>-/-</sup> mice post-MI**

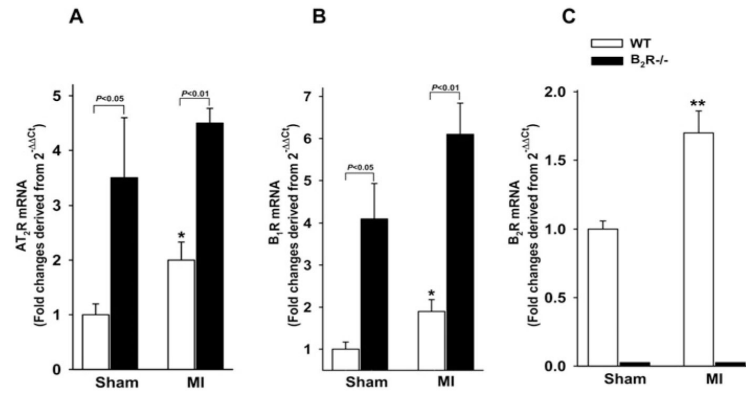
(A) Representative Western blots of pERK1/2 and total ERK1/2 (tERK1/2). (B) Semi-quantitative analysis of pERK1/2 corrected by tERK1/2 and expressed as fold change relative to sham within strain. \* $P < 0.05$  compared with sham within strain; † $P < 0.05$  and †† $P < 0.01$  compared with vehicle within strain; # $P < 0.05$  compared with AT<sub>2</sub>R-ant or B<sub>1</sub>R-ant alone within strain.





**Figure 6. Effect of AT<sub>2</sub>R and/or B<sub>1</sub>R blockade on urinary NO excretion in WT and B<sub>2</sub>R<sup>-/-</sup> mice post-MI**

†† $P < 0.01$  and ††† $P < 0.001$  compared with vehicle within strain; # $P < 0.05$  compared with AT<sub>2</sub>R-ant or B<sub>1</sub>R-ant alone within strain.



**Figure 7. Effect of  $B_2R$  deletion on  $AT_2R$  and  $B_1R$  mRNA expression evaluated by real-time RT (reverse transcription)–PCR and normalized to GAPDH**  
Results are presented as the fold change relative to sham WT controls. \* $P < 0.05$  and \*\* $P < 0.001$  compared with sham within strain.

**Table 1**  
**Effect of blockade of AT<sub>2</sub>R and/or B<sub>1</sub>R on SBP, HR, tissue weight and infarct size in WT and B<sub>2</sub>R<sup>-/-</sup> mice post-MI**

\* $P < 0.05$ , \*\* $P < 0.01$ , compared with sham MI within strain; † $P < 0.05$  and †† $P < 0.01$  compared with WT; # $P < 0.05$  compared with vehicle within strain. LVW, LV weight corrected by body weight; THW, total heart weight corrected by body weight; lung and liver weight were corrected by body weight; IS, infarct size.

Parameter	WT					B <sub>2</sub> R <sup>-/-</sup>				
	MI					MI				
	Sham MI (n=13)	Vehicle (n=9)	AT <sub>2</sub> R-ant (n=10)	B <sub>1</sub> R-ant (n=7)	AT <sub>2</sub> Rant+ B <sub>1</sub> R-ant (n=8)	Sham MI (n=12)	Vehicle (n=9)	AT <sub>2</sub> R-ant (n=9)	B <sub>1</sub> R-ant (n=7)	AT <sub>2</sub> Rant+ B <sub>1</sub> R-ant (n=9)
BW (g)	31±2	31±2	31±1	31±1	31±1	33±11	32±1	31±1	35±1	33±2
SBP (mmHg)	111±4	106±5	110±4	108±5	106±4	123±5	120±7	111±3	119±4	117±5
HR (beats/min)	673±12	642±25	670±14	646±20	673±23	654±18	618±19	601±29	661±15	626±31
LVW (mg/10 g)	31±1	41±1**	38±2	39±2	38±2	35±2†	42±2**	41±3	44±3	43±3
THW (mg/10 g)	42±2	53±2**	51±3	52±2	51±3	47±2†	58±4*	56±4	60±4	58±4
Lungs (mg/10 g)	60±4	61±4	70±6	60±7	61±5	60±2	67±7	71±7	68±4	70±4
Liver (mg/10 g)	392±18	381±14	409±11	364±22	384±15	443±18	420±15	454±25	469±10††#	463±14††#
IS (%)	-	34±3	32±3	34±3	32±2	-	37±3	38±3	36±4	36±3