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Role of Bradykinin B$_2$ and B$_1$ Receptors in the Local, Remote, and Systemic Inflammatory Responses That Follow Intestinal Ischemia and Reperfusion Injury$^1$

Danielle G. Souza,* Eliane S. L. Lopez,‡⁺ Vanessa Pinho,* João Bosco Pesquero,§ Michael Bader,¶ Jorge Luís Pesquero,† and Mauro M. Teixeira$^2*$

The administration of bradykinin may attenuate ischemia and reperfusion (I/R) injury by acting on B$_2$Rs. Blockade of B$_2$R has also been shown to ameliorate lesions associated with I/R injury. In an attempt to explain these contradictory results, the objective of the present work was to investigate the role of and interaction between B$_1$ and B$_2$ receptors in a model of intestinal I/R injury in mice. The bradykinin B$_2$R antagonist (HOE 140) inhibited reperfusion-induced inflammatory tissue injury and delayed lethality. After I/R, there was an increase in the expression of B$_2$R mRNA that was prevented by HOE 140. In mice that were deficient in B$_2$Rs (B$_2$R$^{-/-}$ mice), inflammatory tissue injury was abrogated, and lethality was delayed and partially prevented. Pretreatment with HOE 140 reversed the protective anti-inflammatory and antilethality effects provided by the B$_1$R$^{+/+}$ phenotype. Thus, B$_2$Rs are a major driving force for B$_2$R activation and consequent induction of inflammatory injury and lethality. In contrast, activation of B$_1$Rs may prevent exacerbated tissue injury and lethality, an effect unmasked in B$_1$R$^{-/-}$ mice and likely dependent on the vasodilatory actions of B$_2$Rs. Blockade of B$_1$Rs could be a more effective strategy than B$_2$ or B$_1$/B$_2$ receptor blockade for the treatment of the inflammatory injuries that follow I/R. The Journal of Immunology, 2004, 172: 2542–2548.

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inins are vasoactive peptides that have been implicated in several inflammatory conditions (1–6). The effects of these peptides may be direct or associated with the stimulation of secondary mediators of inflammation, including prostanooids, tachykinins, cytokines, mast cell-derived products, and NO (7, 8). Kinins, of which bradykinin appears to be the most important member, act on two distinct receptors: B$_2$ and B$_1$ receptors (7, 9, 10). B$_2$R is constitutively expressed on various cell types, including endothelial cells, nerve fibers, leukocytes, and mast cell (8, 11). In contrast, there is little B$_1$R expression in most tissues, and its expression may be induced or enhanced by cytokines in stressful situations, such as shock and inflammation (12, 13).

The results of a number of recent studies indicate that the administration of bradykinin at relatively low doses attenuates ischemia and reperfusion (I/R)$^{17}$ injury, especially in the heart (14–18). These protective effects appear to be secondary to the potent vasodilatory effects of bradykinin on ischemic and/or reperfused vessels. However, we have recently demonstrated that, in a model of intestinal I/R in rat, the treatment with HOE 140, a specific B$_2$R antagonist, partially ameliorated lesions associated with reperfusion injury (19). These results were justified on the basis of the well-known proinflammatory actions of bradykinin (8), which include the expression of adhesion molecules, facilitation of leukocyte infiltration, and formation of interendothelial gaps and protein extravasation in postcapillary venules (2, 8, 20–22). The activation of B$_2$Rs is also capable of inducing the expression of B$_1$Rs (23), suggesting that novel B$_1$R expression and activation could participate in the cascade of events leading to tissue injury and systemic inflammation after reperfusion of an ischemic vascular territory. The objective of the present work was to investigate the role of and interaction between B$_1$ and B$_2$ receptors in a model of intestinal I/R injury in mice. Initial experiments confirmed a role for bradykinin B$_1$Rs during I/R injury by using HOE 140, a well-known antagonist at B$_2$Rs. Then, a series of experiments was conducted in mice that were deficient in B$_1$Rs (B$_1$R$^{-/-}$) and that were treated or not with the B$_2$R antagonist.

Materials and Methods

Animals

B$_2$R$^{-/-}$ mice were generated as previously described (24). B$_2$R$^{-/-}$ male C57BL/6 × sv129 mice (10–12 wk) and their wild-type littermate controls were housed under standard conditions and had free access to commercial chow and water. All procedures described in this study had prior approval from the local animal ethics committee.

Ischemia and reperfusion

Mice were anesthetized with urethane (140 mg/kg, i.p.), and laparotomy was performed. The superior mesenteric artery (SMA) was isolated, and ischemia was induced by totally occluding the SMA for 60 min. For measuring percentage of surviving mice, reperfusion was re-established, and mice were monitored for the indicated time periods. For the other parameters, reperfusion was allowed to occur for 30 min when mice were sacrificed. This time of reperfusion (30 min) was chosen based on the presence

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$^3$ Abbreviations used in this paper: I/R, ischemia and reperfusion; SMA, superior mesenteric artery; MPO, myeloperoxidase; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemotactic protein-1.
of significant tissue injury without unduly high mortality rates. Sham-operated animals were used as controls. HOE 140 or vehicle (PBS) was administered (i.v.) 10 min before reperfusion.

**Evaluation of changes in vascular permeability**

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability, as previously described (25, 26). Evans blue (20 mg/kg) was administered i.v. (1 ml/kg) via a tail vein 2 min before reperfusion of the ischemic artery. Thirty minutes after reperfusion, a segment of the duodenum (~3 cm) was cut open and allowed to dry in a petri dish for 24 h at 37°C. The dry weight of the tissue was calculated, and Evans blue was extracted using 1 ml of formamide (24 h at room temperature). The amount of Evans blue in the tissue was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in a plate reader. Evans blue of per 100 mg of tissue. The right ventricle was flushed with 10 ml of PBS to wash the intravascular Evans blue in the lungs. The left lung was then excised and used for Evans blue extraction. The right lung was used for the determination of myeloperoxidase (MPO) as described below.

**MPO concentrations**

The determination of hemoglobin concentrations in tissue was used as an index of tissue hemorrhage. After washing the intestines to remove excess blood, a sample of ~100 mg of duodenum was removed and homogenized in Drabkin’s color reagent according to instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). The suspension was centrifuged for 15 min at 9000 g and filtered using 0.2-μm filters. The resulting solution was read using an ELISA plate reader at 520 nm and compared against a standard curve of hemoglobin.

**Measurement of hemoglobin concentrations**

The determination of hemoglobin concentrations in tissue was used as an index of tissue hemorrhage. After washing the intestines to remove excess blood, a sample of ~100 mg of duodenum was removed and homogenized in Drabkin’s color reagent according to instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). The suspension was centrifuged for 15 min at 9000 g and filtered using 0.2-μm filters. The resulting solution was read using an ELISA plate reader at 520 nm and compared against a standard curve of hemoglobin.

**Measurement of cytokine/chemokine concentrations in serum, intestine, and lungs**

The concentration of cytokines—TNF-α, IL-1β, IL-10, and IL-18—and chemokines—keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein-1 (MCP-1)—in samples was measured in serum and tissue of animals using commercially available Abs and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN). Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at −20°C until further analysis. Serum samples were analyzed at a 1/3 dilution in PBS. One hundred milligrams of duodenum or lung of sham-operated and reperfused animals were homogenized in 1 ml of PBS (0.4 M NaCl and 10 mM NaPO₄) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 Ki aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000 × g, and the supernatant was used immediately for ELISA at a 1/3 dilution in PBS.

**Expression of B₁ R mRNA**

The methodology used was similar to that previously described by Campos et al. (29). Samples of intestine (100 mg) were collected after I/R and frozen under liquid nitrogen. The samples were then homogenized, and the total RNA was extracted using the TRIZol reagent (Life Technologies, Grand Island, NY). One microgram of total RNA was reverse transcribed using oligo(dT) as primer (25 μg/ml), and 200 U of reverse transcriptase (Life Technologies), in 20 μl of PCR buffer containing the following: 0.5 mM dNTP, 10 mM DTT, 2.5 mM MgCl₂, 30 mM KCl, and 20 mM Tris-HCl (pH 8.3) (25). The samples were incubated for 50 min at 42°C, heated for 15 min at 70°C, and cooled in ice. After treatment with 2 U of RNase H (20 min, 37°C), CDNA amplification of a specific sequence of mouse B₁ R and β-actin was performed by PCR using the following primers: sense, 5′-AACCCGTTCACCTGGCC-3′; and antisense, 5′-GACATAAATC TGGTTCT-3′ for B₁ R, and sense, 5′-CGAGGCGGACACAGAGAG-3′; and antisense, 5′-CACTGCGGATCTCTTCTT-3′, for mouse β-actin. β-actin CDNA was used for standardization of the amount of RNA. Five microliters of reverse-transcribed aliquots was mixed in a 20 mM Tris-HCl buffer (pH 8.4) containing the following: 1.5 mM MgCl₂, 300 μM dNTP, 2 μg/ml each primer, and 50 U/ml Taq polymerase (Life Technologies), in a final volume of 100 μl. The cycling protocol used was the following: 4 min at 94°C, 36 cycles of 35 s at 94°C, 45 s at 60°C, and 45 s at 72°C; and finally, 7 min at 72°C. Aliquots of 25 μl were analyzed on a 20% TBE (Tris/borate/EDTA) polyacrylamide gel and stained by ethidium bromide. The size of products is 646 bp for B₁ R and 536 bp for β-actin.

**Drugs and reagents**

The following drugs were obtained from Sigma-Aldrich (St. Louis, MO): uropoietin B, Evans blue, hexadecyltrimethylammonium bromide, and 3,3,5,5-tetramethyl-benzidine. HOE 140 was a gift from Dr. K. Wirth (Hoechst, Frankfurt, Germany). HOE 140 was dissolved in PBS just before use and used at 1 mg/kg, a dose previously shown to effectively block B₂ R (19).

**Statistical analysis**

Results are shown as the mean ± SEM. Percent inhibition was calculated by subtracting the background levels of Evans blue extravasation or MPO (obtained in sham-operated animals) from control and treated animals. Differences were evaluated by using ANOVA followed by Student-Newman-Keuls posthoc analysis. Results with a p < 0.05 were considered significant.

**Results**

Role of B₂ R during I/R injury

The first series of experiments was performed with the aim of confirming the role of B₂ Rs in a model of intestinal I/R in mice. In this model, in addition to the severe local (intestine) and remote (lung) tissue injury, as assessed by the increase in vascular permeability, neutrophil influx, hemorrhage, and release of cytokines, there is much systemic inflammation, as seen by systemic concentrations of cytokines and significant lethality (28). Treatment with HOE 140 (1 mg/kg) significantly prevented the increase of vascular permeability and neutrophil recruitment in the intestine and lungs of reperfused animals (Fig. 1, a–d). Similarly, the B₂ R antagonist markedly prevented intestinal hemorrhage (Fig. 1e). Furthermore, postischemic treatment with HOE 140 partially prevented the reperfusion-induced increases in TNF-α concentrations in the intestine, lungs, and serum (Fig. 2). These results are in marked agreement with our previous study in rats demonstrating that HOE 140 was also capable of diminishing the inflammatory injury and delaying lethality following intestinal I/R (19). In addition, we also assessed in this study the effects of HOE 140 treatment on the production of IL-1 family cytokines (IL-1β and IL-18) and chemokines (KC and MCP-1). After reperfusion injury, there was a significant production of IL-1β, IL-18, KC, and MCP-1 in the intestine and lungs of mice (Table 1). HOE 140 prevented the production of IL-18, KC, and MCP-1 concentrations but induced a further increase in the release of IL-1β release (Table 1). HOE 140 treatment also greatly potentiated the release of IL-10 in the intestines and lungs after intestinal I/R injury (Fig. 3). The inhibition of tissue and systemic TNF-α production was mirrored by a significant delay of lethality in HOE 140-treated mice (Fig. 4). It is of note that all animals eventually died within 2 h after reperfusion (Fig. 4).

Role of B₁ R during I/R injury

As B₁ Rs may be expressed in response to several inflammatory stimuli, including B₁ R activation (8, 13), we evaluated whether this receptor was induced in our model. As seen in Fig. 5 (compare lanes 1 and 2 to lanes 3 and 4), there was a significant expression of B₁ R mRNA in the intestine and lungs 30 min after reperfusion of the ischemic mesenteric artery. To evaluate the functional role of these B₁ Rs in our model, reperfusion injury was performed in B₁ R−/− mice. Akin to the treatment with the B₁ R
antagonist, there was a complete prevention of tissue injury, as assessed by the increase in vascular permeability, neutrophil recruitment, and hemorrhage, in reperfused B$_1$R$^{-/-}$ mice (Fig. 1). In the latter mice, the reperfusion-induced increases in TNF-$\alpha$ production in tissue and serum were also greatly suppressed (Fig. 2). There was also a significant suppression of the production of IL-18, KC, and MCP-1 in reperfused B$_1$R$^{-/-}$ mice when compared with their wild-type controls (Table I). In general, the inhibitory phenotype observed in B$_1$R$^{-/-}$ mice was greater than or, at least, similar to that seen in mice treated with HOE 140 (see Fig. 2 and Table I). Similarly to the situation observed in HOE 140-treated mice, the concentrations of both IL-10 (Fig. 3) and IL-1$\beta$ (Table I) were significantly enhanced in B$_1$R$^{-/-}$ mice when compared with reperfused wild-type mice. In addition to the observed suppression of tissue and systemic inflammation, there was a significant delay in reperfusion-induced lethality in B$_1$R$^{-/-}$ (Fig. 6). Delay in lethality was accompanied by a partial prevention of lethality, because a fraction of animals were alive until 240 min of reperfusion (Fig. 6).

**Effects of the concomitant inhibition of both B$_2$ and B$_1$ receptors**

The next series of experiments were conducted to evaluate whether B$_2$R activation was a relevant stimulus for B$_1$R up-regulation and whether concomitant inhibition of both receptors would enhance the protective effects observed with either treatment alone. The former was indeed the case because inhibition of B$_2$Rs with HOE
Results are shown as picograms of the cytokine per 100 mg of tissue and are the mean ± SEM of at least six animals in each group. *p < 0.01, when compared with sham-operated animals; #, p < 0.01, when compared with vehicle-treated wild-type animals submitted to I/R; and §, p < 0.01, when compared with vehicle-treated B1R−/− mice. ND, Not detected.

FIGURE 2. Reperfusion-associated increase in the concentrations of TNF-α in the intestine, lungs, and serum of wild-type and B1R−/− mice. TNF-α was measured using specific ELISA. In wild-type or B1R−/− mice, HOE 140 was administered i.v. at the dose of 1.0 mg/kg 10 min before reperfusion. Results are shown as picograms of the cytokine per 100 mg of tissue and are the mean ± SEM of at least six animals in each group. *p < 0.01, when compared with sham-operated animals; #, p < 0.01, when compared with vehicle-treated wild-type animals submitted to I/R; and §, p < 0.01, when compared with vehicle-treated B1R−/− mice. ND, Not detected.

To evaluate the concomitant inhibition of both receptors, B1R−/− mice received a systemic injection of HOE 140 before the reperfusion. As seen in Fig. 1, the pretreatment of B1R−/− mice with HOE 140 failed to prevent the local (intestine) and remote (lungs) tissue injury observed in reperfused untreated wild-type mice. It is of note that concomitant inhibition of both B1 and B2 receptors actually prevented the suppressive effects observed when either receptor was blocked alone. A similar situation is observed when the concentration of inflammatory cytokines (TNF-α and IL-18) and chemokines (KC and MCP-1) is measured, i.e., lack of inhibition when both the antagonist and the B1R−/− phenotype are present (Fig. 2, Table I). Furthermore, in B1R−/− mice treated with HOE 140, there was no enhancement of the concentrations of IL-10 or IL-1β, as seen when either receptor is blocked alone (Fig. 3, Table I). Consistent with the results above, treatment of B1R−/− mice with HOE 140 failed to affect significantly the reperfusion-associated lethality observed in untreated wild-type mice (Fig. 6). Indeed, the concomitant functional absence of both receptors induced a lethality that was significantly greater than when either receptor was blocked/absent alone (Figs. 4 and 6).

Discussion

We have recently demonstrated that following intestinal I/R injury in rats, there is an increase in tissue kallikrein activity and activation of B2Rs (19). Prevention of B2R activation with receptor antagonists was accompanied by amelioration of tissue injury and partial prevention of lethality (19). Our first objective was to confirm a role for B2Rs in a model of intestinal I/R in mice. Similarly to the experiments in rats, pretreatment of mice with HOE 140, a selective B2R antagonist, markedly inhibited the reperfusion-associated neutrophil recruitment, increase in vascular permeability, and release of TNF-α. In addition, we show that HOE 140 prevented the reperfusion-induced elevation of chemokines (KC and MCP-1) and IL-18 in mice. The inhibitory effects were observed both in the intestine and lungs of reperfused animals. Moreover, treatment with the B2R antagonist augmented the reperfusion-induced release of IL-10 and partially prevented lethality in both species. Thus, it is clear that B2R activation plays an important role in the cascade of events leading to local (intestine), remote (lungs), and systemic injury following intestinal I/R. These results are consistent with other studies demonstrating an important role of bradykinin in acute inflammatory responses (1–6). The mechanisms by which B2R activation facilitates inflammatory injury appear to be multiple. Thus, activation of B2Rs may directly induce leukocyte migration and degranulation, and bradykinin may also act on leukocytes and/or endothelial cells to release proinflammatory mediators, such as cytokines and platelet-activating factor (8, 30, 31).

Thus, we investigated whether B1R mRNA was being expressed in the intestine and lungs of reperfused mice (Fig. 5, compare lanes 3 and 4 to lanes 5 and 6).

Several studies have now shown that B1R activation or mediators released (e.g., TNF-α) during the reperfusion process may enhance B1R expression during inflammatory responses (34–36). Thus, we investigated whether B1R mRNA was being expressed and played a functional role in cooperation with B2Rs in mediating injury in our system. Initial experiments showed marked B1R mRNA expression in the lungs and intestine following I/R of the SMA in mice (see Fig. 5). This is in line with other studies demonstrating the expression of functional kinin B1Rs in the endothelium after I/R (37, 38). In support of an important functional role for the B1Rs being expressed, experiments in B1R−/− mice showed that local and remote inflammatory injury was markedly suppressed in receptor-deficient mice when compared with their wild-type counterparts. Thus, there was no increase in vascular permeability, neutrophil accumulation, and hemorrhage in the intestine and lungs of reperfused B1R−/− mice. Moreover, the reperfusion-associated tissue increase of TNF-α, IL-18, MCP-1, and KC was suppressed in B1R−/− mice when compared with wild-type mice. Additionally, serum concentrations of TNF-α were partially suppressed, and this was associated with a significant delay in reperfusion-associated lethality in B1R−/− mice. Not only was lethality delayed, but there was also a degree of protection because ~40% of animals were alive after 2 h of reperfusion. These results
strongly suggest that B1R activation plays an important functional role during intestinal I/R tissue injury and demonstrate that B1R activation plays a relevant role for reperfusion-associated inflammation and lethality.

The mechanisms underlying the role for B1R activation in our system are not known at present. However, bradykinin acting via B1Rs may induce NF-kB activation in various cell types (39, 40) and, consequently, induce the release of proinflammatory cytokines. As we have previously demonstrated the essential role of NF-kB activation in various cell types (39, 40), it is possible that interference with the influx of neutrophils is a major driving force for TNF-α production following intestinal I/R injury (42).

Because either treatment with the B2R antagonist or the B1R antagonist afforded marked protection against tissue injury and significant, albeit not complete, protection against lethality, we examined whether concomitant B1 and B2 receptor absence would afford greater protection. To our surprise, the suppression of tissue inflammation observed in B1R−/− mice was almost completely reversed by the treatment with the B2R antagonist. Indeed, in B1R−/− mice that received HOE 140, the inflammatory tissue injury, as assessed by increase in vascular permeability, neutrophil

![Image](http://www.jimmunol.org/...)

**FIGURE 3.** Reperfusion-associated increase in the concentrations of IL-10 in the intestine and lungs of wild-type (WT) and B1R−/− mice. IL-10 was measured using specific ELISA. In wild-type or B1R−/− mice, HOE 140 was administered i.v. at the dose of 1.0 mg/kg 10 min before reperfusion. Results are shown as picograms of the cytokine per 100 mg of tissue and are the mean ± SEM of at least six animals in each group. *p < 0.01 when compared to sham-operated animals; #, p < 0.01 when compared with vehicle-treated WT animals submitted to I/R; and $, p < 0.01 when compared to vehicle-treated B1R−/− mice.

**FIGURE 4.** Reperfusion-associated increase in lethality of wild-type (WT) mice. Survival was monitored as indicated and survivors were sacrificed after 120 min. In WT mice, HOE 140 was administered i.v. at the dose of 1.0 mg/kg 10 min before reperfusion. There were at least 10 animals in each group for the survival experiments.
recruitment, hemoglobin content, or tissue concentration of cytokines, was overall very similar to that observed in untreated wild-type mice and greater than that in animals that were only treated with HOE 140 or only B2R. Even the increase of IL-1β or IL-10 was absent in B1R mice that received HOE 140 treatment. The enhanced tissue inflammation was reflected in greater lethality. As such, B1R mice given the B2R antagonist died significantly more than B2R mice given vehicle. Thus, either B2R blockade or the B1R phenotype has a similar inhibitory effect on tissue inflammation, but the B2R phenotype afforded greater protection against lethality. Treatment of B1R mice with the B2R antagonist reversed all the protection seen in the transgenic mice. One possible explanation for these apparent contradictory results could be that the activation of B2Rs was having two major effects in the system—an effect on tissue cells inducing the expression B2Rs and a protective effect. As for the former effect, there is evidence to suggest that B2R activation takes place at the beginning of the inflammatory process, and that via auto-regulatory mechanisms, the desensitization of B2Rs occurs, followed by the up-regulations of B1Rs. In our experiments, blockade of B2Rs with HOE 140 prevented the up-regulation of B1Rs in the intestine and lungs of reperfused animals. Thus, in this model of I/R injury in the mouse, B2R activation is a major driving force for B1R expression. The results of a number of recent studies indicate that administration of bradykinin at comparatively low doses attenuates I/R injury (14–16). These protective effects could be playing a role in our system and appear to be secondary to the potent vasodilatory effects of bradykinin on B2Rs of ischemic and/or reperfused vessels.

In summary, our results confirm an important role for B2R activation during I/R injury. Moreover, it is shown in this study that the activation of B2Rs has two important functions during intestinal I/R injury. First, B2Rs are a major driving force for B1R activation and consequent induction of inflammatory injury and lethality. In contrast, activation of B1Rs may facilitate tissue perfusion via its vasodilatory effects, thus preventing exacerbated injury. In wild-type mice, the proinflammatory B1R-dependent effects of B,R activation prevail, and blockade of the latter receptor prevents tissue injury and lethality. However, the protective effects of B2Rs are unmasked in B2R−/− mice. In the latter animals, blockade of B2Rs is accompanied by worse tissue injury and enhanced lethality. Thus, although the activation of B1Rs is necessary for the up-regulation of B1R and consequent acute proinflammatory effects, B2 activation also leads to vasodilatation and protection against reperfusion injury. Together, the present data highlight the interaction between B1 and B2 receptors in a relevant pathophysiological in vivo model and suggest that blockade of B1Rs could be a more effective strategy than B2R or B1R/B2R blockade for the treatment of the inflammatory injuries that follow the reperfusion of an ischemic vascular bed.

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