Stimulation of the Beta2 Adrenergic Receptor at Reperfusion Limits Myocardial Reperfusion Injury via an Interleukin-10-Dependent Anti-Inflammatory Pathway in the Spleen

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Background: In addition to the airway-relaxing effects, β2 adrenergic receptor (β2AR) agonists are also found to have broad anti-inflammatory effects. The current study was conducted to define the role of β2AR agonists in limiting myocardial ischemia/reperfusion injury (IRI).

Methods and Results: Adult male wild-type (WT) and interleukin (IL)-10 knockout (KO) mice underwent a 40-min left coronary artery ligation and 60-min reperfusion. A selective β2AR agonist, Clenbuterol, at doses of 0.1 μg or 1 μg/g weight i.v. 5 min before reperfusion, significantly reduced myocardial infarct size (IS) by 28% and 39% (vs. control, P<0.05) in WT mice respectively, but had no protective effect in IL-10 KO mice. Inhalational therapy with nebulized Clenbuterol, Albuterol, Salmeterol or Arformoterol immediately before ischemia significantly reduced IS (P<0.05) in WT mice. Splenectomy similarly reduced IS as Clenbuterol-treated mice, but intravenous Clenbuterol did not further reduce IS in splenectomized mice. In splenectomized WT mice, acute transfer of isolated splenocytes, not the Clenbuterol-pretreated splenocytes, restored the myocardial IS to the level of intact mice. Intravenous Clenbuterol significantly increased splenic protein levels of β2AR, phosphorylated Akt and IL-10 and plasma IL-10, and inhibited the expression of pro-inflammatory mRNAs.

Conclusions: Both intravenous and inhalational β2AR agonists exert a cardioprotective effect against IRI by activating the anti-inflammatory β2AR-IL-10 pathway.

Key Words: β2AR; Clenbuterol; Heart; Ischemia/reperfusion; Myocardial infarction

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**Animals and Experimental Protocols**

C57BL/6 wild type (WT) mice (9–13 weeks of age, purchased from The Jackson Laboratory) and congenic interleukin (IL)-10 knockout (KO) mice (9–13 weeks of age, breeding pairs purchased from The Jackson Laboratory) were randomly assigned to either IR injury groups or sham surgery groups. Compared to the WT mice, the phenotype of IL-10 KO mice showed significantly higher spleen weight (% of body weight, 0.446±0.033% vs. 0.392±0.013%, P<0.05). There was no difference in body weight, heart weight, blood pressure and heart rate. There was no evidence of dermatitis or rectal prolapse as reported in the literature.

In treated mice, Clenbuterol (purchased from Sigma-Aldrich, St. Louis, MO, USA) was administered either as an i.v. bolus 5 min before reperfusion at 2 different doses, 0.1 µg/g and 1.0 µg/g weight with an injection volume of 2µL/g body weight, or nebulized in a solution with 1 mg Clenbuterol in 5 mL saline. Clenbuterol was nebulized using an Ecosonic nebulizer (Medisonic USA Inc., Clarence, NY, USA). The effect of Clenbuterol on heart rate was determined using PowerLab instrumentation (ADInstruments, Colorado Springs, CO, USA). Additional 3 short-acting to long-acting β2AR agonists, albuterol, salmeterol and Arformoterol (purchased from Sigma-Aldrich), were also tested individually in 3 groups of mice and administered by inhalation before ischemia.

**Myocardial IR Injury and Measurement of IS**

In previous work, we established that myocardial IS as measured by late-gadolinium-enhanced MRI at 60 min of reperfusion attains 95% of the size measured by the same method at 24 h post-reperfusion in mice. We therefore used 60 min of reperfusion in the present study. The left coronary artery (LCA) of WT or IL-10 KO mice was ligated for a duration of 40 min followed by 60 min of reperfusion, as detailed previously. Briefly, mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and orally intubated. An additional dose of pentobarbital (40 mg/kg, i.p.) was applied shortly after reperfusion. The adequacy of the anesthesia was confirmed by hind limb pinch reflex every 15 min. The heart was exposed through a left thoracotomy. The LCA was identified under a dissecting microscope. An 8-0 Prolene suture was placed around the LCA at a level 1 mm inferior to the left auricle. Ischemia was induced by securing a suture over a piece of PE-60 tubing placed parallel to the LCA, and reperfusion was achieved by removing the tube. Successful ligation of the LCA was confirmed by blanching in the ischemic zone. The mice were euthanized 60 min after reperfusion, and the explanted hearts were cannulated through the ascending aorta for perfusion with 3 mL of 1.0% TTC (Sigma-Aldrich). The LCA was then re-occluded with the same suture used for coronary occlusion prior to perfusion with 10% Phthalo blue to determine risk region (RR), which was statistically similar among all groups. The left ventricle was then cut into 5–7 transverse slices that were weighed and digitally photographed to determine IS as a percent of RR.

**Splenic Leukocyte Adoptive Transfer**

Splenocytes are purified from WT mice and then transferred into recipient mice 5 min before reperfusion via external jugular vein injection at a dose of 5×10^6 splenocytes cells/mouse in 50 µL. In Clenbuterol-treated splenocytes, the splenocytes were treated with Clenbuterol for 10 min at a dose of 0.01 µg in 20×10^6 splenocytes cells (200 µL). After treatment, these splenocytes were re-suspended in Clenbuterol-free PBS. Splenocytes were counted using a Cellometer (Nexcelom, Lawrence, MA, USA).

Several additional groups of mice without IRI were treated either with PBS or Clenbuterol. The plasma, heart and spleen were harvested at 5, 15, 30 min following treatment. The samples were analyzed for ELISA, RT-PCR and Western Blot analysis. Clenbuterol-induced tachyarrhythmia was evaluated in mice treated with both bolus i.v. injection and nebulization using a PowerLab physiological monitoring system (Colorado Springs, CO, USA).

**Western Analysis**

Protein levels of β2AR, Akt phosphorylation, CD38, FPR1,
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were determined by BCA protein assay (ThermoFisher Scientific, Rockford, IL, USA). Twenty micrograms of protein were separated by 10% SDS-PAGE. After transfer, nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) were probed with primary antibodies against β2AR, total Akt, phosphorylated Akt (anti-Ser473), CD38, FPR1, IL-1β and IL-10 (ThermoFisher Scientific) at a 1:2,000 dilution and with secondary antibodies (Promega, Madison, WI, USA) at a 1:5,000 dilution in blocking solution (0.5% BSA in TBS-T). Proteins were visualized with enhanced chemiluminescent substrate (ThermoFisher Scientific) followed by densitometry using a FluorChem 8900 imaging system (Alpha Innotech, Santa Clara, CA, USA). β-actin was used as a loading control.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

Splen ic mRNA levels of CD38, Eg2, Fpr1, IL-10, IL-1β and Ncf1 were assessed by qRT-PCR. In brief, total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN USA, Germantown, MD, USA) according to the manufacturer’s instructions. cDNAs were synthesized using an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). qPCR was performed

IL-1β and IL-10 in the spleen and/or plasma were assessed by Western blot analysis, as previous described. Briefly, left ventricular and splenic tissue were homogenized in PBS. Plasma samples were obtained by centrifuging blood samples at 1,600 g for 20 min. Total protein concentrations
with the SsoAdvanced™ Universal SYBR Green supermix (Bio-Rad Laboratories) and monitored using a CFX Real-Time PCR Detection System (Bio-Rad Laboratories). GAPDH (Unique Assay ID: qMmuCID0006259 for CD38, qMmuCID0009042 for Egr2, qMmuCID0015439 for Fpr1, qMmuCID0015452 for IL10, qMmuCID0005641 for IL-1β and qMmuCID0006405 for Ncf1). mRNA levels were quantified using the 2(ΔΔCt) relative quantification method, as previously described.15

Statistical Analysis
All data are presented as mean±SEM (standard error of the mean). Changes in heart rates were analyzed using repeated measures ANOVA followed by Bonferroni pairwise comparisons. All other data were compared using one-way ANOVA followed by t-test for unpaired data with Bonferroni correction.

Results
Both C57BL/6 WT mice and congenic IL-10 KO mice underwent 40 min of LCA occlusion followed by 60 min of reperfusion. A long-acting selective β2AR agonist, Clenbuterol, at doses of 0.1 and 1 µg/g weight, was administered as an i.v. bolus 5 min before reperfusion. Clenbuterol at 0.1 and 1 µg/g weight significantly attenuated myocardial IS by 28% and 39% in WT mice respectively (IS as %RR: 39.2±2.8 and 33.4±4.0 vs. control 54.7±3.4, P<0.05). There was no significant difference in the infarct-sparing effect of Clenbuterol between these 2 doses. The infarct-sparing effect of Clenbuterol disappeared in IL-10 KO mice, even at the high dose of 1 µg/g weight. There was no difference in RR among these groups (Figure 1). Inhalational therapy with nebulized Clenbuterol (1 mg in 5 mL normal saline for 5 min immediately before ischemia significantly reduced IS (40.8±4.9 vs. 53.6±3.0, P<0.05) in WT mice (Figure 2A–C). Intravenous bolus administration of Clenbuterol significantly increased the heart rate by 36% in both high-dose and low-dose mice (Figure 3A). However, inhalational therapy with Clenbuterol only increased the heart rate by 11% from the corresponding baseline, a modest effect that did not achieve statistical significance (Figure 3B). Three more selective β2AR agonists were tested to evaluate their roles in attenuating myocardial IRI. Albuterol (short-acting at a dose of 300 µg/mL), salmeterol (moderate-acting at a dose of 20 µg/mL) and arformoterol (long-acting at a dose of 10 µg/mL) were nebulized to treat mice for 1 min before LCA occlusion. All these β2AR agonists exerted similar cardioprotective effect in attenuating myocardial IS (Figure 2D).

Splenectomy before ischemia attenuated IS to a similar extent as in Clenbuterol-treated mice (32.7±4.9 vs. control 53.6±3.0, P<0.05). However, Clenbuterol at a dose of 1 µg/g weight did not provide for any additional reduction in IS in splenectomized mice (Figure 4A). In splenectomized WT mice, splenic leukocytes adoptive transfer (SPAT) from WT mice 5 min before reperfusion restored the myocardial IS to the level of that of intact mice (52.1±3.7 vs. intact WT control 53.6±3.0, P<0.05). Clenbuterol-pretreated splenocytes failed to increase the IS in splenectomized mice (Figure 4B). Without re-suspension of the isolated WT splenocytes, PBS-treated splenocytes had a 30% loss at 10 min and a 34% loss at 30 min, whereas the Clenbuterol-pretreated splenocytes had 15% higher live cells than the untreated splenocytes at both 10 min (P<0.05) and 30 min (P=0.08) (Figure 4C).

In WT mice without IRI, intravenous Clenbuterol at a dose of 1 µg/g weight significantly increased splenic protein...
that treatment with Clenbuterol, a long-acting β2AR agonist, just prior to reperfusion attenuates myocardial IS by inhibiting inflammatory responses in the spleen through the activation of a pathway involving β2AR, Akt and IL-10. Furthermore, our results show that individual inhalational therapy with 4 β2AR agonists with different acting durations exerts similar cardioprotection against IRIs as intravenous treatment. As inhalational therapy has significantly less chronotropic effect, this might enhance its translational potential for patients with acute coronary syndromes.

Recently, several studies have demonstrated that activation of β2AR attenuates myocardial IRI. These studies explore the direct effect of β2AR agonist on cardiomyocytes. However, the anti-inflammatory effects of β2AR in mediating cardioprotection remain to be explored. β2ARs are extensively expressed in the pulmonary system, cardiac muscle and immune cells. Among the adrenergic receptors in immune cells, T and B cells exclusively express β2ARs instead of β1ARs. In immune cells, β2AR stimulation of CD4+ T cells is known to inhibit T cell proliferation via the cAMP-dependent inhibition of nuclear factor κB activation and thus favors a Th2 polarization. β2ARs are also the predominant β-adrenergic receptors in macrophages and neutrophils. β2AR stimulation on macrophages

**Discussion**

Our work and others have demonstrated that the inflammatory response contributes importantly to post-ischemic reperfusion injury in mice. The present study investigated the effects of β2AR activation in inhibiting inflammatory responses and thereby reducing myocardial IS. We found levels of phosphorylated Akt, β2AR and CD38 as early as 5 min post-administration, and these levels stayed elevated for between 5 and 30 min following Clenbuterol treatment (Figure 5A–C). The FPR1 level was significantly reduced following Clenbuterol treatment (Figure 5D). IL-10 levels in the spleen and plasma were only transiently elevated at 5 and 15 min following Clenbuterol treatment, and then returned to normal levels at 30 min following treatment (Figure 6). The IL-1β level was measured but could not be detected in the spleen or the plasma (data not shown).

The qRT-PCR assays demonstrated that Clenbuterol reduced the expression of CD38, Egr2 and Fpr1 mRNAs in splenic tissue at 15 min following treatment, but that CD38 and Fpr1 mRNAs returned to baseline levels by 30 min after treatment. In contrast, Clenbuterol enhanced the expressions of mRNAs for Ncf1, IL-1β and IL-10 at 30 min following treatment (Figure 7).

**Figure 5.** Protein levels of phosphorylated Akt, β2 adrenergic receptor (β2AR), CD38 and FPR1 in the spleen by Western Blot are shown. Levels of phosphorylated Akt, β2AR, CD38 and FPR1 in the spleen were serially evaluated in mice following an i.v. injection of Clenbuterol at a dose of 1.0 µg/g weight. Phosphorylated Akt and β2AR levels were significantly and steadily increased within the 30-min period following treatment. At the same time, levels of CD38 were also significantly elevated in the spleen but started to decline 30 min after treatment. Levels of FPR1 were significantly reduced within the 30-min period following treatment. In the Western blots shown at the top of each graph, n=3 animals per group, except for the 30 min group where n=4 animals. The calculation was based on equal β-lactin levels.

**Figure 6.** mRNA levels of CD38, FPR1 and Ncf1 in the spleen of mice following i.v. injection of Clenbuterol at a dose of 1.0 µg/g weight. The expression of CD38 and FPR1 was significantly reduced at 30 min post-administration, while the expression of Ncf1 was significantly increased at 30 min post-administration.
In the current study, by administering a selective \( \beta_2 \) AR agonist (Clenbuterol) upon reperfusion, we found that Clenbuterol attenuated myocardial IS by 30\%, and its infarct-limiting effect was not due to its effect on cardiomyocytes but rather to its inhibitory effect on inflammatory responses. This conclusion is supported by evidence that: (1) Clenbuterol did not afford additional reduction of myocardial IS in mice without a spleen (Figure 4A); and (2) Clenbuterol did not reduce myocardial IS in IL-10 knockout mice (Figure 1). Splenic leukocytes adoptive transfer before reperfusion restored the myocardial IS in splenectomized mice to the level of that in intact mice. However, Clenbuterol-treated splenocytes, before transfer, abolished this infarct-exacerbating effect of the splenocytes (Figure 4B). As the Clenbuterol washed away from the splenocytes, the systemic role of the Clenbuterol was avoided. The results indicate that the infarct-limiting effect of this \( \beta_2 \) AR agonist is likely mediated by way of the spleen. Clenbuterol enhanced protein expressions of CD38, IL-10 and p-Akt, and decreased expression of FPR1 (Figure 6) supports the notion that activation of \( \beta_2 \) ARs modify the spleen into an anti-inflammatory phenotype. In light of previous work from our lab and others showing that CD4\(^+\) T-cells play an equally critical role in acute reperfusion injury,\(^{3,24,26}\) these results suggest a pathway by which \( \beta_2 \) AR agonists stimulate \( \beta_2 \) ARs on CD4\(^+\) T-cells (probably regulatory T-cells), thus enhancing the release of IL-10, which in turn dampens the activation of immune cells (primarily neutrophils) in the spleen. This pathway then suffices to inhibit the mobilization of neutrophils into the circulatory system, followed by their homing to the infarct zone where they exacerbate IS during reperfusion.

Interestingly, we found that with ex vivo treatment of the splenocytes with Clenbuterol, the reduction in splenocytes was significantly less than that in PBS-treated splenocytes (Figure 4C). Thus, activation of \( \beta_2 \) ARs increased the tolerance of the splenocytes to anoxic injury.

Considerable evidence demonstrates that acute inflammatory responses contribute importantly to reperfusion injury.\(^{12,16}\) Furthermore, the spleen plays a central role in mediating this inflammatory response.\(^{12,16}\) The current study demonstrates that activation of \( \beta_2 \) ARs stimulates a potent anti-inflammatory effect by inhibiting splenic inflammatory responses. As Clenbuterol was administered shortly before reperfusion, the infarct-limiting effect of Clenbuterol is most likely due to its effect in inhibiting inflammatory response during post-ischemic reperfusion, rather than to its effect on the heart or on preconditioning.

A number of clinical studies have included studies on the effect of \( \beta_2 \) AR agonist in patients with ACS.\(^{28-32}\) However, the positive chronotropic effect of \( \beta_2 \) AR agonists might argue against its acute application in the setting of ACS.\(^{40}\) In the present study, we found that intravenous administration of Clenbuterol increased the heart rate by 36\% in mice. This led us to examine the alternative administration route that has previously been used in humans with asthma (inhaledinal administration). Using this approach, Clenbuterol had only a modest effect on heart rate, yet with a comparable reduction in myocardial IS (Figures 1–3). These results raise the question of whether there might be potential for the clinical application of \( \beta_2 \) AR agonists in ACS patients. Towards this end, additional studies may be warranted to investigate the potential of inhalational therapy of \( \beta_2 \) AR agonists against myocardial IRI in animal models of different species, and perhaps human beings. Of note, \( \beta_2 \) AR is the most abundant receptor subtype\(^{41}\) in cardiomyocytes. Activation of \( \beta_2 \) ARs exerts pro-apoptotic effects, while activation of \( \beta_2 \) AR on cardiomyocytes produces preconditioning\(^{4,6}\) and anti-apoptotic effects.\(^{5,32,33}\) In the current study, inhalational therapy of individual Clenbuterol, Albuterol, Salmetorol and Arformoterol was administered before index ischemia. Thus, it is possible that the cardioprotective effects of an inhalational \( \beta_2 \) AR agonist in the current study may involve both the preconditioning of cardiomyocytes and inhibition of pro-inflammatory responses.

Although we found that Clenbuterol inhibited the exacerbation of IS by splenic leukocytes and reduced the expression of pro-inflammatory cytokine mRNAs, such as Egr2 and Fpr1, it also enhanced the expression of other...
pro-inflammatory mRNAs like Ncf1 and IL-1β (Figure 7). We found consistent expression of protein and mRNA in FPR1 and IL-10; however, an opposite trend of changes was found in CD38 and IL-1β (Figures 6, 7). IL-1β protein in the splenic tissue and the plasma was not detectable (data not shown). Of note, all these changes in mRNA levels were transient. It is also important to mention that these results are limited by small sample sizes. The overall net effect of Clenbuterol administration was anti-inflammatory in nature, given the reduction in IS. As both pro-inflammatory and anti-inflammatory cytokine mRNAs were enhanced, it is possible that Clenbuterol might have differential effects on different inflammatory cell populations residing in the spleen.

In summary, using a mouse model of acute myocardial IRI, the present study shows that a selective β2AR agonist, Clenbuterol, attenuates myocardial IS when it is administered just prior to reperfusion. We propose that Clenbuterol inhibits pro-inflammatory responses during post-ischemic reperfusion by promoting the release of IL-10, thereby attenuating the activation of the splenic leukocytes that exacerbate IS upon reperfusion. The bio-effects of Clenbuterol were also evidenced by the overexpression of β2ARs on the splenic leukocytes and activation of pAkt via IL-10. Mice treated with inhalational Clenbuterol have significantly less positive chronotropic effect than with intravenous Clenbuterol, but show a similar attenuation in myocardial IS. These results underscore the potential for β2AR agonists in inhibiting the pro-inflammatory responses that contribute importantly to reperfusion injury. The study also has potential clinical implications because it suggests the possibility of using inhalational β2AR agonists to reduce IS in patients suffering acute myocardial infarction.

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**Conflict of interests**

None.

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