

Endogenous cannabinoids contribute to remote ischemic preconditioning via cannabinoid CB₂ receptors in the rat heart

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Abstract

In addition to well-known neurobehavioral effects, endogenous cannabinoids exert diverse cardiovascular actions. Recently, they have been suggested to protect the myocardium against ischemia/reperfusion injury. The aim of this study is to examine the contribution of endogenous cannabinoids to cardioprotection afforded by remote ischemic preconditioning. Three groups of remote preconditioned (15 min of mesenteric artery occlusion followed by 15 min of reperfusion) and three groups of sham-operated rats were included in the study. Animals were pretreated intravenously by vehicle, cannabinoid CB₁ (AM251, 1 mg/kg) or CB₂ (AM630, 1 mg/kg) receptor antagonist 15 min prior to remote preconditioning or sham operation. Myocardial injury was induced by 30 min of coronary artery occlusion followed by 2 h of reperfusion. The resultant arterial hypotension, ventricular arrhythmias, and infarct size were compared among the groups. Remote preconditioning exerted potent cardioprotection manifested as significant reductions in infarct size ($P < 0.001$) as well as number and duration of arrhythmias ($P < 0.01$, 0.01 and 0.05 for premature ventricular contractions, ventricular tachycardias and fibrillations; respectively). The cannabinoid CB₁ receptor antagonist pretreatment had no significant effect on ischemia-induced hypotension, arrhythmias or infarct size. On the other hand, the cannabinoid CB₂ receptor antagonist pretreatment abolished the protective effects of remote preconditioning on infarct size ($P < 0.01$) and arrhythmias ($P < 0.01$), without any significant effect on ischemia-induced hypotension. The results of this study suggest that endogenous cannabinoids, through acting on cannabinoid CB₂ receptors, are involved in the cardioprotective phenomenon of remote ischemic preconditioning, induced by mesenteric artery occlusion and reperfusion.

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1. Introduction

Remote ischemic preconditioning or preconditioning at a distance is a cardioprotective phenomenon which was first described by Przyklenk et al. (1993) in the canine model of myocardial ischemia/reperfusion. In their report, brief episodes of ischemia in one vascular bed protected remote virgin myocardium from subsequent sustained coronary occlusion (intra-cardiac remote preconditioning). A similar adaptive phenomenon, inter-organ remote preconditioning, has also been reported which is triggered by ischemia/reperfusion of other organs such as kidneys

(Takaoka et al., 1999), intestines (Wolfrum et al., 2002), and limbs (Kharbanda et al., 2002; Kristiansen et al., 2005; Weinbrenner et al., 2002, 2004) in a variety of other species. Despite the promising results of the preliminary explorations of the clinical application of this clinically feasible procedure (Gunaydin et al., 2000), the mechanisms responsible for this phenomenon and its signaling pathways are still remained elusive.

In addition to their well-known neurobehavioral effects (Howlett et al., 2004), endogenous cannabinoids exert important physiological roles in cardiovascular system, such as hypotension and bradycardia (Randall et al., 2002, 2004; Wagner et al., 1998). These effects are considered to be due to activation of specific G-protein-coupled, cannabinoid CB₁ and CB₂ receptors (Niederhoffer and Szabo, 1999). Although cannabinoid CB₁ receptors are preferentially located on brain,

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the cardiovascular depressor effects of cannabinoids appear to mostly involve cannabinoid CB₁ receptors expressed in peripheral tissues, including blood vessels, the heart, and sympathetic nerve terminals (Batkai et al., 2004). The possible contribution of cannabinoid CB₂ receptors, which are mainly located on peripheral non-neuronal cells (Niederhoffer and Szabo, 1999) including cardiovascular system, to modulation of cardiovascular function is less well documented.

Recent investigations have proposed the involvement of endogenous cannabinoids in infarct size-reducing effects conferred by lipopolysaccharide (Lagneux and Lamontagne, 2001) and heat stress (Joyeux et al., 2002), as well as endothelial protection afforded by classic ischemic preconditioning (Bouchard et al., 2003) in isolated rat hearts. However, the roles of endogenous cannabinoids in infarct size-reducing and anti-arrhythmic effects of remote ischemic preconditioning have not yet been evaluated. Therefore, we examined the cardioprotective effects of endogenous cannabinoids in the well-known model of remote preconditioning induced by a brief episode of mesenteric artery occlusion and reperfusion in intact rats.

2. Materials and methods

2.1. Animals

Animals were handled in accordance with the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (NIH US publication 85-23 revised 1996). Male Sprague–Dawley rats weighing 200–250 g were used. Animals were housed in groups of 3–4 in a room controlled at 22±1°C and maintained in an alternating 12-h light/12-h dark cycles, and were allowed free access to food and water.

2.2. Study groups and experimental protocols

Animals were randomly divided into 6 groups:

1. *Vehicle + sham operation*: Vehicle was intravenously administered 15 min before mesenteric artery isolation and manipulation, followed by myocardial ischemia/reperfusion protocol.
2. *Vehicle + remote preconditioning*: Vehicle was intravenously administered 15 min before mesenteric artery occlusion (15 min) and reperfusion (15 min) prior to myocardial ischemia/reperfusion protocol.
3. *Cannabinoid CB₁ receptor antagonist + sham operation*: The cannabinoid CB₁ receptor antagonist (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, AM251, Tocris, 1 mg/kg) was intravenously administered 15 min before sham operation.
4. *Cannabinoid CB₁ receptor antagonist + remote preconditioning*: AM251 (1 mg/kg) was intravenously administered 15 min before mesenteric artery occlusion.
5. *Cannabinoid CB₂ receptor antagonist + sham operation*: The cannabinoid CB₂ receptor antagonist (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone, AM630, Tocris, 1 mg/kg) was intravenously administered 15 min before sham operation.

6. *Cannabinoid CB₂ receptor antagonist + remote preconditioning*: AM630 (1 mg/kg) was intravenously administered 15 min before mesenteric artery occlusion.

Patel et al. (2002) have previously demonstrated that the cardioprotective effect of a single 15-min episode of mesenteric artery occlusion/reperfusion is more substantial than the standard ischemic preconditioning protocol of 3 cycles of 5-min occlusion/reperfusion. Therefore, remote preconditioning was produced according to this protocol in the present study.

2.3. General surgical procedure

Animals were anesthetized via intraperitoneal administrations of sodium pentobarbital (50 mg/kg; Merck, Darmstadt, Germany). Trachea was exposed through a ventral midline cervical incision, and intubated with a cannula connected to a rodent ventilator. Rats were ventilated with room air at 60 breaths/min. Atelectasis was prevented by maintaining a positive end-expiratory pressure of 2 cmH₂O. The carotid artery and jugular vein were cannulated for measurement of arterial blood pressure (Pressure Transducer Model P-1000-A, Narco Biosystem, Houston, TX, USA) and intravenous drug injections, respectively. Lead II of electrocardiogram was recorded using subcutaneous needle electrodes and cardiac coupler connected to a DMP-4B physiograph (Narco Biosystems, Houston, TX, USA). Abdominal incision was made to expose the superior mesenteric artery, which was either manipulated or occluded via a vascular clip as mentioned above (*Study groups and experimental protocols*), after a steady state was achieved. Left thoracotomy was performed ≈ 15 mm from the sternum to expose the heart at the level of the 4th ribs. The pericardium was removed and the left atrial appendage was moved to reveal the location of the left coronary artery. The vein descending along the septum of the heart was used as the marker for the left coronary artery. A ligature (6–0 prolene) along with a snare occluder was placed around the vein and left coronary artery close to the place of origin. Coronary artery was occluded for 30 min, confirmed by epicardial cyanosis, decreased blood pressure and increased R wave amplitude in electrocardiography. Reperfusion, for a period of 2 h, was achieved by loosening the snare.

2.4. Evaluation of arrhythmia

Premature ventricular contractions, ventricular tachycardia, consecutive run of at least four premature ventricular contractions, and ventricular fibrillation were the main observed arrhythmias. In this study, the number of premature ventricular contractions as well as duration of ventricular tachycardia and ventricular fibrillation were determined and compared among the groups. The arrhythmia analysis was limited to the ischemic period.

2.5. Determination of infarct size

After 2 h of reperfusion the coronary artery was re-occluded. The area at risk was determined by negative staining. Methylene blue (10%) was administered via the jugular vein to effectively

stain the non-occluded area of the left ventricle. The rat was sacrificed and the heart was excised. The left ventricle was removed from the remaining tissue and subsequently cut into 2-mm cross sectional pieces. This allowed for the delineation of the normal area, stained blue, versus the area at risk that subsequently remained pink. The area at risk was excised from the non-ischemic area, and the tissues were placed in separate vials containing 1% 2,3,5-triphenyltetrazolium chloride (Sigma, St Louis, MO, USA) in 100 mM phosphate buffer (pH=7.4) for 15 min to indicate viable (brick red color) and nonviable (gray) tissues. Tissues were then fixed in vials of 10% formaldehyde overnight to enhance the contrast between viable and necrotic tissue. Digital photographs were taken (Sony digital camera, Model DSC-T1, Japan) and infarct size and area at risk were determined using planimetric technique (AutoCAD version 11.0, CA, USA). The results are expressed as infarct size/area at risk (%).

2.6. Statistics

Data are expressed as mean±S.E.M. The results were analyzed by analysis of variance (ANOVA) followed by Tukey's HSD as post-hoc test to compare the means. A value of $P<0.05$ was considered significant.

3. Results

3.1. Hemodynamic parameters

As presented in Table 1, there was no significant difference in basal hemodynamic parameters among the groups. Variations in mean arterial pressure, as the percentage of the basal mean arterial pressure, is summarized in Fig. 1. AM251, but not AM630, treatment resulted in significant mean arterial pressure elevation in both sham-operated and preconditioned groups compared to the basal levels ($P<0.05$). Mean arterial pressure was significantly elevated by mesenteric artery occlusion ($P<0.01$) and returned to the basal level by reperfusion. Mean

Table 1
Basal heart rate (beats/min) and mean arterial pressure (mm Hg) and 30 min after myocardial ischemia

Experimental group	Basal		30 min after myocardial ischemia	
	Heart rate (beats/min)	Mean arterial pressure (mm Hg)	Heart rate (beats/min)	Mean arterial pressure (mm Hg)
Sham-operated (vehicle)	421±9	107±8	405±8	74±7 ^a
Remote preconditioned (vehicle)	413±11	104±7	397±12	63±8 ^a
Sham-operated (AM251)	426±8	113±5	412±10	84±8 ^a
Remote preconditioned (AM251)	423±12	109±6	418±11	76±6 ^a
Sham-operated (AM630)	434±11	116±7	422±9	73±7 ^a
Remote preconditioned (AM630)	416±7	105±5	408±9	65±8 ^a

^a $P<0.001$ compared with corresponding basal group.

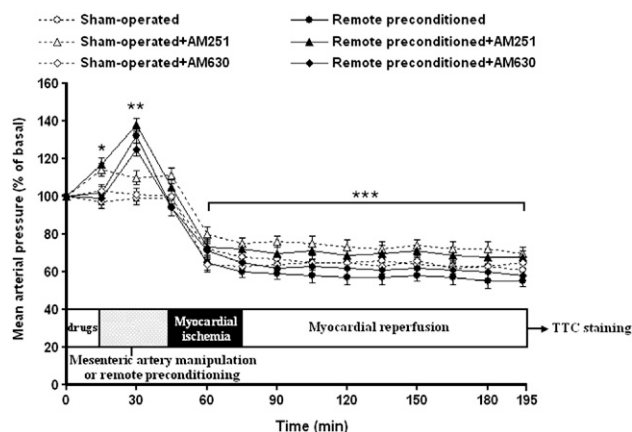


Fig. 1. Mean arterial pressure (MAP) changes during the experimental protocol in animals treated with intravenous injections of vehicle, CB₁ receptor antagonist (AM251, 1 mg/kg) or CB₂ receptor antagonist (AM630, 1 mg/kg). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ after Tukey's HSD post-hoc test (compared to the corresponding basal values).

arterial pressure changes during mesenteric artery occlusion and reperfusion were not significantly affected by AM251 or AM630 administration. Myocardial ischemia resulted in significant hypotension ($P<0.001$), which persisted constant during myocardial reperfusion. Neither AM251 nor AM630 was able to prevent ischemia/reperfusion-induced hypotension ($P<0.001$, compared to their basal level; $P>0.05$ compared to the corresponding vehicle-treated group). In addition, as shown in Table 1, there was no significant difference in hemodynamic parameters among the groups 30 min after myocardial ischemia.

3.2. Arrhythmias

The incidence of ventricular tachycardia and fibrillation among the experimental groups is shown in Table 2. The arrhythmia analysis is limited to ischemic period. There were only a limited number of arrhythmias following reperfusion which were not included in the results and were not significantly different among the groups. As it is demonstrated in Fig. 2, remote preconditioning led to a significant reduction in the number of premature ventricular contractions ($P<0.01$) as well as durations of ventricular tachycardia ($P<0.01$) and ventricular fibrillation ($P<0.05$). Neither AM251 nor AM630 had a significant effect on ischemia/reperfusion-induced arrhythmias in sham-operated animals. However, the ameliorating effect of

Table 2
Incidence of ventricular tachycardia and fibrillation among the experimental groups

Experimental group	Ventricular tachycardia	Ventricular fibrillation
Sham-operated (vehicle)	100%	100%
Remote preconditioned (vehicle)	100%	75%
Sham-operated (AM251)	100%	100%
Remote preconditioned (AM251)	100%	62.5%
Sham-operated (AM630)	100%	100%
Remote preconditioned (AM630)	100%	100%

remote preconditioning on arrhythmias was abolished by AM630 pretreatment ($P < 0.01$). AM251 pretreatment had no significant effect on the anti-arrhythmic effect of remote preconditioning.

3.3. Infarct size

The area at risk was not significantly different among the groups (data not shown). As it is demonstrated in Fig. 3, remote preconditioning resulted in a marked reduction in infarct size/area at risk ($P < 0.001$). This infarct size-reducing effect of remote preconditioning was not affected by AM251 pretreatment, but was abolished by AM630 pretreatment ($P < 0.01$,

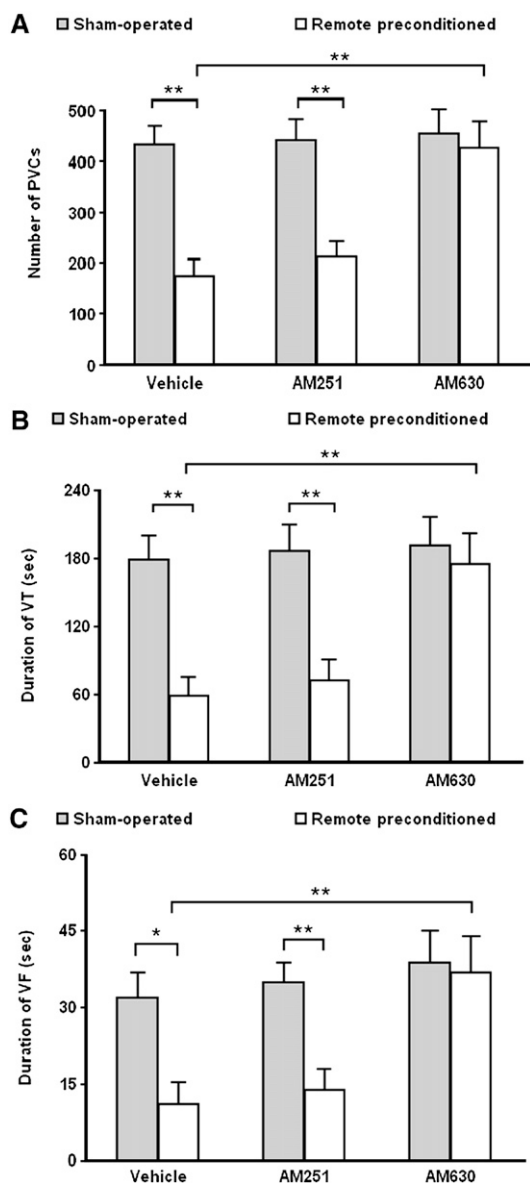


Fig. 2. The number of (A) premature ventricular contractions (PVCs), (B) duration of ventricular tachycardia (VT) and (C) ventricular fibrillation (VF) in sham-operated and remote preconditioned rats treated with intravenous injections of vehicle, CB₁ receptor antagonist (AM251, 1 mg/kg) or CB₂ receptor antagonist (AM630, 1 mg/kg). * $P < 0.05$, ** $P < 0.01$ after Tukey's HSD post-hoc test.

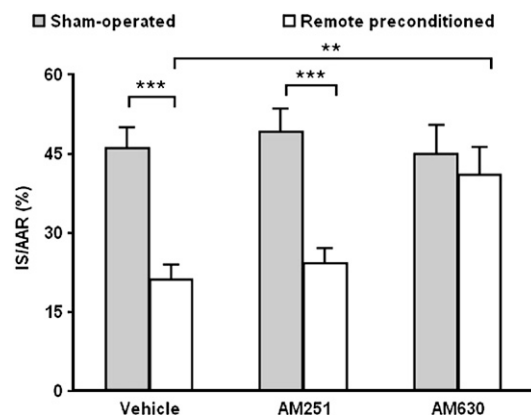


Fig. 3. Infarct size/area at risk (IS/AAR) in sham-operated and remote preconditioned rats treated with intravenous injections of vehicle, CB₁ receptor antagonist (AM251, 1 mg/kg) or CB₂ receptor antagonist (AM630, 1 mg/kg). ** $P < 0.01$, *** $P < 0.001$ after Tukey's HSD post-hoc test.

compared to vehicle-treated remote preconditioning group). AM251 and AM630 treatment had no significant effect on infarct size/area at risk in sham-operated animals.

4. Discussion

This study provides evidence that endogenous cannabinoids contribute to the cardioprotective effects of remote preconditioning, conferred by a brief episode of mesenteric artery occlusion and reperfusion. This cardioprotection, manifested as reduction in the infarct size and severity of arrhythmias, seems to be mediated through cannabinoid CB₂ receptors.

In this study we used AM251 as a specific cannabinoid CB₁ receptor antagonist. AM251 is structurally very close to SR 141716A (rimonabant) which is the prototypic member of a series of compounds named diarylpyrazole cannabinoid CB₁ receptor antagonists. These ligands readily prevent or reverse effects mediated by CB₁ receptors (Lan et al., 1999). AM251 has been shown to be a potent and selective cannabinoid CB₁ receptor antagonist which is 306-fold selective over cannabinoid CB₂ receptors. In addition, AM251 is about two-fold more selective for the CB₁ receptor when compared to SR 141716A (Lan et al., 1999). We also used AM630 as a specific cannabinoid CB₂ receptor antagonist. Several studies have demonstrated that AM630 is a potent cannabinoid CB₂ receptor antagonist which is 165-fold selective over CB₁ receptors (Hosohata et al., 1997a,b).

Many endogenous neurotransmitters, peptides and hormones have been proposed to play roles in the signal transduction pathways mediating the cardioprotective effects of ischemic preconditioning. It has been hypothesized that a variety of triggers, either receptor-dependent (adenosine, opioids, prostaglandins, etc.) or receptor-independent (nitric oxide, free oxygen radicals, calcium, etc.), may result in the activation of K_{ATP} channels, which are thought to be the end effectors of ischemic preconditioning (Eisen et al., 2004). For instance, using the nonselective adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (8-SPT) and the K_{ATP} channel blocker sodium 5-hydroxydecanoate Pell et al. (1998) demonstrated that activation of both adenosine receptors and K_{ATP} channels

appears to be involved in acute renal preconditioning of the rabbit myocardium. However, there are still many uncertainties regarding different aspects of signal transduction pathways of either classic or remote ischemic preconditioning.

Production of endogenous cannabinoids (palmitoylethanolamide and 2-arachidonylglycerol) (Schmid et al., 2000), as well as expression of their receptors (Galiegue et al., 1995; Gebremedhin et al., 1999; Liu et al., 2000) in the cardiovascular system, suggested them as possible modulators of cardiovascular function (Wagner et al., 1997, 2001, 2003). While recent investigations regarding cardiovascular effects of endogenous cannabinoids provide evidence of their involvement in development of hemodynamic complications in several pathological states (Batkai et al., 2001; Ros et al., 2002; Varga et al., 1998; Wagner et al., 1997, 2001, 2003), they have also been proposed as endogenous cardioprotective agents (Bouchard et al., 2003; Di Filippo et al., 2004; Joyeux et al., 2002; Lagneux and Lamontagne, 2001). In the following sections, we are mainly focused on these two aspects.

4.1. Ischemia/reperfusion-induced hemodynamic instability

In the first few hours after myocardial infarction cardiogenic shock, characterized by inadequate cardiac output, profound hypotension and systemic hypoperfusion, is a common clinical problem, and shows a grave prognosis with high rates of in-hospital mortality (Wagner et al., 2001). Involvement of endogenous cannabinoids in the ischemia-induced hypotension was first reported by Wagner et al. (2001), in the anesthetized rat model of myocardial ischemia/reperfusion. In addition to the ability of endogenous cannabinoids to induce hypotension following exogenous administration (Randall et al., 2002, 2004), they have also been reported to contribute to development of hypotension in pathologic conditions such as hemorrhagic (Wagner et al., 1997) and septic (Varga et al., 1998) shock, as well as hemodynamic abnormalities and hyperdynamic circulation of cirrhosis (Ros et al., 2002; Batkai et al., 2001). In all these conditions, endogenous cannabinoids have been thought to mediate hypotension mainly by acting on peripheral vascular CB₁ receptors, which were blocked by cannabinoid receptor antagonist, SR-141716 (Batkai et al., 2001; Ros et al., 2002; Varga et al., 1998; Wagner et al., 1997, 2001, 2003). However, the CB₁-mediated hypotension in the above-mentioned pathologic states is now a matter of debate. Recent evidence indicates the existence of cannabinoid receptors distinct from CB₁ or CB₂ that are inhibited by SR-141716 but not by other more selective CB₁ receptor antagonists such as AM251 (Batkai et al., 2004). Batkai et al. (2004) using different cannabinoid receptor antagonists (SR-141716 and AM251) as well as transgenic animals have recently provided strong evidence that receptors distinct from CB₁ or CB₂ are involved in endotoxemia-induced hypotension. In another study, Ford et al. (2002) have shown that one or more novel sites, distinct from either cannabinoid CB₁ or CB₂, may mediate coronary vasodilatory and negative inotropic responses to endocannabinoids such as anandamide. According to the results of the present study, AM251 was not able to prevent ischemia/reperfusion-induced hypotension. This

finding along with the reported contribution of endogenous cannabinoids to ischemia/reperfusion-induced hypotension (Wagner et al., 2001), suggests that receptors other than CB₁ or CB₂ might also be involved in development of hemodynamic instability following myocardial ischemia.

4.2. Ischemia/reperfusion-induced arrhythmia and necrosis

This study confirms that a brief episode of mesenteric artery occlusion and reperfusion can protect the heart against ischemia/reperfusion-induced ventricular arrhythmias and myocardial necrosis. There is evidence that exogenous administration of cannabinoids reduces infarct size (Di Filippo et al., 2004), attenuates arrhythmias (Krylatov et al., 2002), and enhances functional recovery following ischemia/reperfusion injury (Lepicier et al., 2003), through CB₂ receptors. By contrast, there is evidence that the infarct-limiting action of the endocannabinoid anandamide was not mimicked by ACPA (the selective cannabinoid CB₁ receptor agonist) or JWH133 (the selective cannabinoid CB₂ receptor agonist) either used individually or in combination (Underdown et al., 2005). Furthermore, it was shown that this action of anandamide was blocked by the presence of either rimonabant or SR144528, which are regarded as CB₁ and CB₂ receptor selective antagonists, respectively, suggesting that the endocannabinoids limit the cardiac infarction associated with ischemic/reperfusion by activation of one or more novel cannabinoid sites of action (Underdown et al., 2005). However, the endogenous production of cannabinoids has also been suggested to contribute to the infarct size-reducing effect and improvement of functional recovery in the setting of endogenous cardioprotective mechanisms, such as classic ischemic preconditioning (Bouchard et al., 2003), heat stress (Joyeux et al., 2002) and lipopolysaccharide-induced cardioprotection (Lagneux and Lamontagne, 2001). However, contribution of endogenous cannabinoids to anti-arrhythmogenic effect of ischemic preconditioning has not yet been evaluated. The results of this study suggest that endogenous cannabinoids are involved in the ameliorating effects of ischemic preconditioning on ischemia/reperfusion-induced arrhythmias. It also clarifies for the first time that remote ischemic preconditioning is mediated through cannabinoid CB₂ receptors.

Remote ischemic preconditioning is triggered by ischemia/reperfusion of a variety of organs. The model used in the present study was selected since mesenteric ischemia/reperfusion with the resultant ischemia of gastrointestinal system is widely used and well standardized in intact rat studies (Patel et al., 2002). Productions of endogenous compounds, such as opioids, in the gastrointestinal system and their subsequent transportations to the heart have been postulated to exert the cardioprotective effects of this protocol (Patel et al., 2002). Endogenous cannabinoids are produced in the gastrointestinal system and play important roles in the physiological control of its function, such as motility (Mascolo et al., 2002). The endogenous cannabinoid system has also been reported to be physiologically involved in the protection against excessive inflammation in the gastrointestinal system (Massa et al., 2004). The findings of our study along with the presence of cannabinoid receptors in the

myocardium (Galiegue et al., 1995; Gebremedhin et al., 1999; Liu et al., 2000), suggest that the production of endogenous cannabinoids could be among the mechanisms responsible for the cardioprotection induced by mesenteric artery occlusion and reperfusion. However, the potential roles of endogenous cannabinoids in induction of cardioprotection by means of clinically more feasible protocols of remote preconditioning need to be further studied.

In summary, the findings of this study provide evidence of the contribution of endogenous cannabinoids to cardioprotection induced by a brief episode of mesenteric artery occlusion and reperfusion. These cardioprotective effects of endogenous cannabinoids on ischemia/reperfusion-induced arrhythmia and necrosis are mediated by cannabinoid CB₂ receptors.

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