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Original Paper

Activation of Cannabinoid Type 2 Receptor by JWH133 Protects Heart Against Ischemia/Reperfusion-Induced Apoptosis

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Key Words
Cannabinoid type 2 receptor • Ischemia/reperfusion • Apoptosis • Heart

Abstract
Background: Cannabinoid type 2 (CB2) receptor agonists can protect myocardium against ischemia/reperfusion (I/R) injury although the underlying mechanism remains unclear. Here we report the antiapoptotic effect of CB2 receptor agonist, JWH133, during myocardial ischemia/reperfusion injury and potential underlying mechanisms. ***Methods:*** Ischemia was performed by blocking left coronary artery of rat for 30 min. After ischemia for 30 min, the rat heart was reperfused for 120 min by loosing the ligation of blocking left coronary artery. JWH133 (20 mg/kg), a CB2 receptor selective agonist, or vehicles were injected intravenously 5 minutes before ischemia. Infarct size of myocardium was assessed by histological stain, myocardial apoptosis index (AI) was determined by TUNEL, and mitochondrial membrane potential ($\Delta\Psi m$) was measured by flow cytometry. Western blots were performed to measure the cytochrome *c* release, cleaved caspase 3, cleaved caspase 9 and PI3K/Akt kinase phosphorylation. ***Results:*** JWH133 significantly reduced the infarct size and AI of myocardium suffering I/R compared to vehicle-treated group. Further mechanistic study revealed that activation of CB2 receptor by JWH133 inhibited the loss of $\Delta\Psi m$, reduction of the cleaved caspases-3 and -9, release of mitochondrial cytochrome *c* to the cytosol, and increase of phosphorylated Akt. These JWH133-mediated effects could be totally abrogated by PI3K inhibitor wortmanin or CB2 receptor antagonist AM630. ***Conclusion:*** Our results demonstrate that activation of CB2 receptor by JWH133 prevent apoptosis during ischemia/reperfusion through inhibition of the intrinsic mitochondria-mediated apoptotic pathway and involvement of the PI3K/Akt signal pathway.

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Introduction

Endocannabinoid system, which comprises specific cannabinoid receptors, endogenous ligands (endocannabinoids), and synthetic and degradative pathways [1], is a new therapeutic target in variety of disorders, such as inflammation and tissue injury, including cardiovascular system [2]. Among of them, at least two types of cannabinoid (CB) receptors, CB1 and CB2 receptor, have been found and cloned [3, 4]. It has been reported that CB1 and CB2 receptors are widespread in many tissues including cardiac myocyte [5, 6]. Emerging evidence suggests that CB2 receptor elicits protective effects during early steps of ischemia/reperfusion (I/R) injury. While incubation with CB2 agonists, infarct size is reduced either before ischemia or during reperfusion *ex vivo or in vivo* [1, 7-9]. Consistently with these results, blockade of CB2 receptors eliminates cardiac protective effect of endocannabinoids in rat isolated hearts exposed to low-flow ischemia and reperfusion [10, 11]. Furthermore, a single dose of the CB2 receptor agonist, JWH-133, can reduced infarct size of myocardium [12]. The mechanistic investigations reveal that the protective effect of CB2 receptor may ascribe to reduction of cardiac leukocyte recruitment, reduction of superoxide generation, or increase of ERK 1/2 and STAT-3 phosphorylation.

Apoptosis, a genetically controlled programmed cell death, has been found to play a critical role during I/R injury in mammals. Cardiomyocyte apoptosis has been shown exist in different cardiac disease of mammals [13, 14], such as ischemic heart disease, heart failure and I/R. As apoptosis or its underlying "caspase activation" mechanism can cause necrosis, caspase inhibitors are used to reduce infarct size after I/R [15, 16]. Recent studies reported that CB2 receptor was involved in antiapoptotic effect during I/R, and, CB2 receptor agonist JWH-133 could reduce cardiomyocyte apoptosis [17]. Many studies show that mitochondria plays important roles in apoptosis: (a) they supply ATP that is necessary for execution of apoptosis; (b) they release cytochrome *c* and apoptosis-inducing factor proteins that are involved in caspase activation and nuclear fragmentation [18]. By far, it is still uncertain whether antiapoptotic effect of CB2 receptor is related to mitochondria pathway.

Here we evaluated the antiapoptotic effect of the CB2 selective agonist JWH133 in myocardial I/R rat model. Importantly, we investigated the mechanisms underlying the antiapoptotic effect of JWH133. We demonstrated that JWH133 inhibited the mitochondria-mediated apoptotic pathway during I/R injury, in which process PI3K/Akt signal pathway might be involved. This work provided mechanistic insights into the antiapoptotic function of CB2 receptor in heart I/R.

Materials and Methods

Animals

Experiments were carried out in adult male Sprague-Dawley rats (weighting 230-280g) obtained from the Experimental Animal Center of Hebei Province. This study was performed conforming to Guide for the Care and Use of Laboratory Animals described by Directive 2010/63/EU of the European Parliament. Animal work was approved by the Ethics Committee for Animal Experiments of the Hebei Medical University, in compliance with NIH, and carried out in compliance with China government guidelines. In this experiment, total of 66 rats were used. There were 5 groups, and each experimental group had 12 rats. In addition, 6 rats were excluded for technical or other reasons.

In vivo I/R injury of the rat heart

Rats were anesthetized with pentobarbital (150 mg/kg, i.p.) and the body temperature was maintained at 37.0°C± 0.5°C. Animals were ventilated with a rodent ventilator (HX-300S, Chengdu TME Technology Co Ltd., China) at 60 to 70 breaths per minute with tide volume of about 15 ml/kg. Electrocardiogram (ECG) in lead II together with the blood pressure of carotid artery were continuously monitored and recorded using a data acquisition system (PowerLab/8 s, AD Instrument, Australia). Left thoracotomy was performed in the 3rd or 4th intercostals space, and pericardium was opened to expose the heart. A 5/0 silk suture was passed

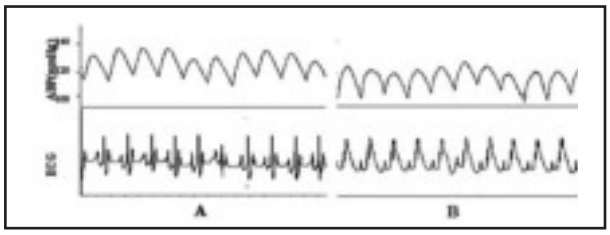

Fig. 1. Arterial blood pressure (ABP) and Electrocardiogram (ECG) before and after ligation of the left descending coronary. A: ABP and ECG before ligation of the left descending coronary. B: ABP and ECG after ligation of the left descending coronary.

around the left descending artery (LDA). After stabilization of cardiac function for 15 min, myocardial ischemia was produced by ligating LDA and reperfusion was produced by loosing the ligation [19]. Classical ischemic sign following coronary arterial occlusion was indicated by a significant ST-segment elevation in ECG immediately after LDA ligation, together with a slight blood pressure reduction (Fig. 1). The coronary artery was occluded for 30 minutes followed by 120-minute reperfusion, 1000 units of sodium heparin were given intravenously before coronary artery occlusion. Sham-operated rats were treated with the same surgical protocol as described but without occlusion. At the end of reperfusion, half of the rats in each group were sacrificed and left ventricles were taken out for infarct size determination. The other half of the rats in each group were sacrificed and the left ventricular tissues including ischemic and non-ischemic areas were randomly separated into several parts for TUNEL, western blot and flow cytometric measurements.

In vivo drug treatment

JWH133 and the CB2 antagonist AM630 were obtained from Enzo Life Sciences Ltd. (UK), and JWH133 and AM630 were dissolved in DMSO. Rats were randomly assigned into groups to receive JWH133 at a dose of 20 mg/kg or vehicle DMSO 5 min before ischemia by intravenous injection. The selectivity of this dose of JWH133 was in accordance with the report of Montecucco et al. [12]. Some rats were pretreated with AM630 (1 mg/kg) or PI3K inhibitor wortmannin (15 μg/kg, Sigma Corp., USA) by intravenous injection 30 min before injection of JWH133 onset. This dose of wortmannin was in accordance with our previous report [20].

Determination of area at risk (AAR) and infarct size (I)

At the end of reperfusion, both aorta and LDA of rats were ligated completely and 2% Evans blue (1 ml, Sigma Corp., USA) was injected to the heart via left free ventricular wall for delineation of area at risk. Then the heart was removed quickly and frozen. After removal of atrium and right ventricle, the left ventricle was sectioned into 2 mm transverse sections from apex to base (5 slices/heart). Following defrosting, the slices were incubated at 37 °C with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 min, fixed in 10% formaldehyde solution and photographed with a digital camera (Canon Inc., Japanese) to distinguish clearly red stained viable tissue and unstained necrotic tissue. Normal myocardium stained by Evans and TTC looked blue, ischemic myocardium that was not infarct stained by TTC looked red, and infarct myocardium unstained by either Evans or TTC looked pale. Area at risk included red area and pale area. The different zones were determined using image processing system (JIE DA-108, Jiangsu China). Area at risk (AAR) and left ventricular infarct zone (I) were expressed as percentage of ventricle surface (AAR/V) and area at risk (I /AAR), respectively.

TdT-mediated dUTP in situ nick-end labeling (TUNEL)

The left ventricle tissue samples were obtained at the end of the reperfusion, fixed in 10% paraformaldehyde, paraffin-embedded, and sectioned. TUNEL staining was performed in deparaffinized and rehydrated sections according to the demands of TUNEL assay kit (In Situ Cell Apoptosis Detection Kit I, POD). After TUNEL staining, the sections were counterstained with hematoxylin. Total cardiomyocytes and TUNEL positive cells in the specimens were counted by light microscopic analysis. Five high-power fields (×200) of each section were randomly selected. The ratio of TUNEL-positive cells to total cardiomyocytes was apoptosis index (AI).

Measurement of mitochondrial membrane potential (ΔΨm)

To prepare mitochondria for measuring ΔΨm by flow cytometry at the end of reperfusion, the left ventricular myocardium of rats that underwent I/R was excised and rapidly minced followed by

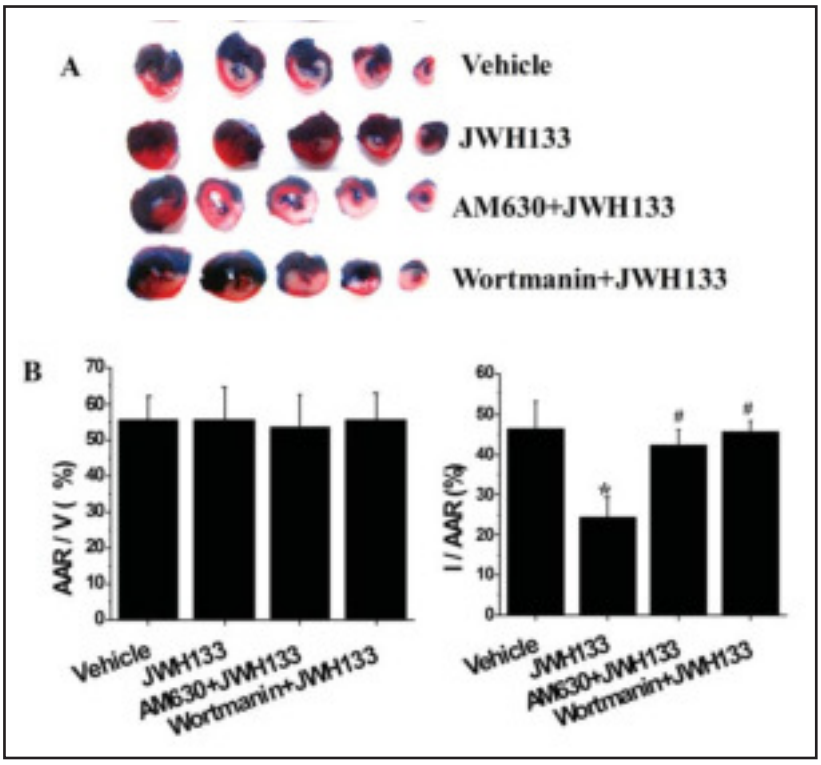

Fig. 2. Administration of CB2 agonist JWH133 (20 mg/kg) reduced infarct size of heart suffering ischemia/reperfusion (I/R) injury. (A) Representative images of stained heart slices. (B) Quantification of area at risk (AAR) per ventricle surface (V) and infarct size (I) per AAR. Vehicle or JWH-133 was administrated 5 min before ischemia, and the CB2 antagonist AM630 (1mg/kg) or PI3K inhibitor wortmanin (15μg/kg) was given 30 min before administration of JWH133 onset. Data were expressed as mean±SEM, n=6 for each group. *P<0.05 vs. vehicle group, #P<0.05 vs. JWH133 group.

homogenization with a Polytron homogenizer (low setting 3) and then with a Potter homogenizer (5 up and down strokes at 1500 rpm) in 15 ml of a cold buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.04 mM fatty acid-free bovine serum albumin, pH 7.4 at 4°C. Homogenates were centrifuged at 1000*g* for 5 min, and supernatants were centrifuged again at 10,000*g* for 10 min. After mitochondrias were collected by centrifugation, mitochondrias were stained with 1μM Rhodamine 123 for 30 min and analyzed by BD FACSCalibur System (Becton Dickinson, USA.).

Isolation of cytosolic fractions from rat heart

To measure cytochrome *c* release from mitochondria, the left ventricular myocardium of rats was excised and rapidly minced followed by homogenization with a Polytron homogenizer and then with a Potter homogenizer (5 up and down strokes at 1500 rpm) in 15 ml of cold buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH=7.4 at 4°C. Homogenates were centrifuged at 1000*g* for 5 min, and supernatants were centrifuged again at 10,000*g* for 10 min. The final supernatants, corresponding to the cytosolic fractions, were collected, and a protease inhibitor phenylmethanesulfonyl fluoride 1 mM (Sigma Corp., USA) was added. The supernatants were immediately frozen at -80°C until determination of protein concentration and analysis of cytochrome *c* with western blot technique.

Western blot

Samples of cytosolic proteins for cytochrome *c* assay were obtained by above method. Proteins from whole cells of left ventricle after reperfusion were extracted in lysis buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.05% SDS, 10 mM NaF, 1 mM PMSF, 2 mM Na_3VO_4, and complete protease inhibitor cocktail tablet (Roche Corp., Swiss). Proteins (50 μg per lane) were electrophoresed through polyacrylamide/SDS gels and transferred by electroblotting onto PVDF membranes. Membranes were blocked for 1 h in 5% (w/v) nonfat milk before incubation with appropriate dilutions of cytochrome *c* antibody (Epitomics Inc., USA), cleaved caspase 3 and cleaved caspase 9 antibodies (Cell Signaling Technology Inc., USA), Akt and p-Akt antibodies (Bioworld Technology Inc., USA) as well as corresponding secondary antibodies. The blots were developed using the ECL system (Immobilion™ Western, Millipore) and were analyzed by Quantity One Software (Bio-Rad, U.S.A.). Protein contents were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Santa Curz) level.

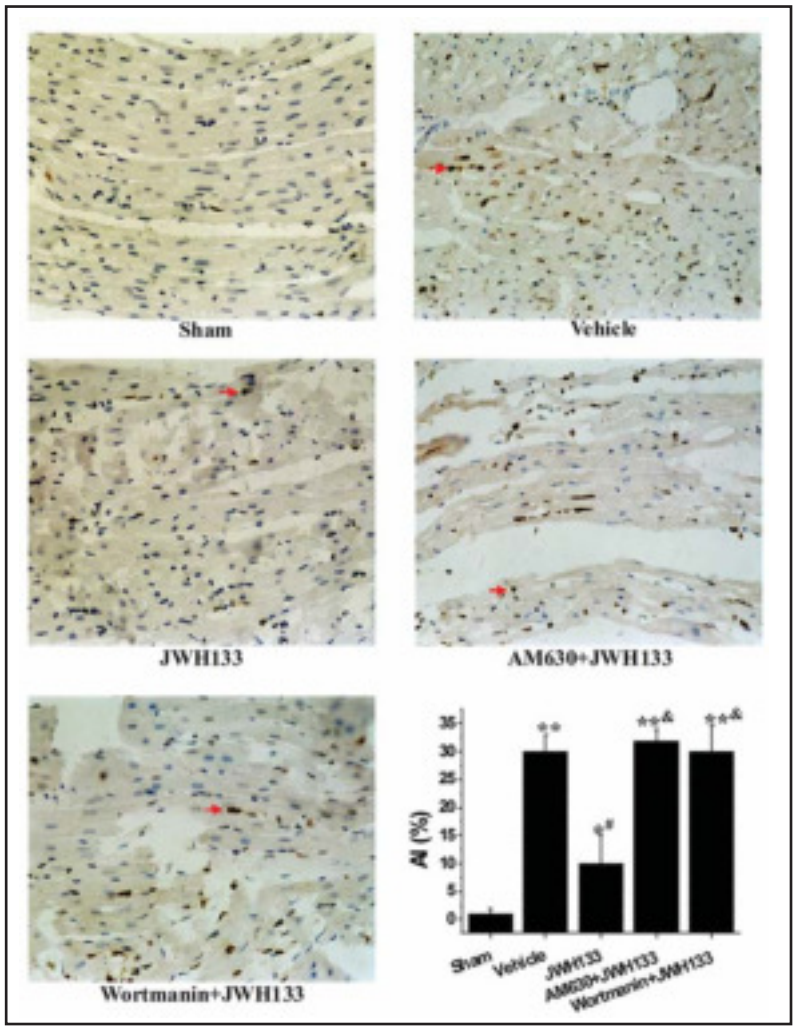

Fig. 3. Effect of JWH133 on apoptosis index of myocardium after 120 min reperfusion was analysed by Tunnel. Representative images were shown. Quantification of the apoptosis index (AI) which was the ratio of TUNEL-positive cells to total cardiomyocytes. Data were expressed as mean±SEM. n=6 in each group. *P<0.05, **P<0.01 vs. Sham group, #P<0.05 vs. vehicle group, &P<0.05 vs. JWH133 group. Photoes were TUNEL stain ×200, and arrows in photoes indicated TUNEL positive cells.

Statistics

Data were expressed as mean ± SEM. The differences of the parameters between prior and posterior to drug application were analyzed by paired Student's t test. Differences between groups were evaluated by one-way ANOVA followed by Dunnet's post hoc test. Statistical significance was accepted at P <0.05.

Results

JWH133 reduced cardiac injury in I/R rats

To test the therapeutic function of selective CB2 receptor activation, rats were treated with CB2 receptor agonist JWH133 (20 mg/kg) or vehicle before ischemia. The histological evaluation revealed an area at risk (AAR) of approximately 55% in the various treatment groups. The infarct size was significantly smaller in JWH133 treated rats (24.6%±4.6 of AAR) compared to vehicle treated rats (46.7%±6.6 of AAR). The effect of JWH133 was inhibited by CB2 receptor antagonist AM630 (1 mg/kg) or PI3K inhibitor wortmannin (15 μg/kg) (Fig. 2). The AM630 or wortmannin alone had no effect on I/R or vehicle-treated rats (data were not shown). These results suggested that CB2 receptor agonist JWH133 could reduce cardiac injury in I/R rats.

JWH133 reduced apoptosis index (AI) in I/R rats

Cytoplasm of TUNEL-positive cells was shrinked but plasma membrane was integral, furthermore the nucleus were pyknotic and marginated to the periphery of cell membrane,

Group	Rhodamine 123 fluorescence
Sham	198.3±10.6
Vehicle	130.6±5.8*
JWH133	187.2±7.5#
AM630+JWH133	125.8±6.9*&
Wortmanin+JWH133	128.8±3.9*&

Table 1. Changes of mitochondrial membrane potential (ΔΨm) detected by flow cytometry in ventricular myocardium suffering ischemia/reperfusion. Data were expressed as mean ± SEM. n=6 for each group. *P<0.05 vs. Sham group; # P<0.05 vs. Vehicle treated group; &P<0.05 vs. JWH133 treated group.

which indicated the condensation of chromatin (Fig. 3). The apoptosis index (AI), the ratio of TUNEL-positive cells to total cardiomyocytes, was significantly increased after 120 min reperfusion in vehicle treated rats as compared to the sham rats (30.1±3.0% vs 1.1±0.8%, P<0.01). JWH133 reduced AI significantly after reperfusion (AI=10.1±5.0%), which was inhibited by pretreatment of AM630 (AI=31.9±2.1%) or wortmannin (AI=29.9±4.6%) (Fig. 3). These results suggested that JWH133 could protect myodicardium against I/R inducing-apoptosis.

JWH133 prevented the loss of mitochondrial membrane potential (ΔΨm) in I/R rats

ΔΨm is an important parameter of mitochondrial function and is used as an indicator of cell health. Rhodamine 123, a cellpermeant, cationic and mitochondrion-selective fluorescent dye which can be washed out of the cells once ΔΨm is lost. Thus, quantification of the fluorescence intensity of Rhodamine 123 has been validated as a measure of ΔΨm [21]. Consistent with previous reports [22], the loss of ΔΨm was induced by reperfusion after ischemia. As shown in Table 1, JWH133 prevented the loss of ΔΨm after reperfusion, and this effect was reversed by AM630 or wortmannin. These results suggested that JWH133 prevented the loss of ΔΨm in I/R rats.

Expression of cleaved caspases-3 and-9, p-Akt, Akt, and release of mitochondrial cytochrome c

As shown in Figure 4, cleaved caspases-3, -9 and the release of mitochondrial cytochrome *c* were significantly increased in the vehicle treated rats (P < 0.05 vs. sham group). These phenomenons could be reversed by JWH133 (P < 0.05 vs. vehicle treated group), which was inhibited by AM630 or wortmannin. We next to examined that the total Akt expression had no difference in all rats. While compared to the vehicle treated rats, the p-Akt expression was significantly increased in the JWH133 treated rats (P < 0.05). Similarly AM630 and wortmannin could reverse the effect of JWH133 (Fig. 4). These results suggested that JWH133 might protect myocardium against I/R through mitochondrial-dependent pathway.

Discussion

Accumulating evidence demonstrated that CB2 receptors play protective roles during I/R injury *ex vivo or in vivo* [1, 7-9]. JWH-133, a CB2 receptor agonist, has been recently proposed to reduce infarct size of myocardium during I/R injury [12]. However, the underlying mechanisms of the protective effect of CB2 receptor are not yet clarified. In the present study, we found that CB2 receptor agonist JWH133 reduced the infarct size through

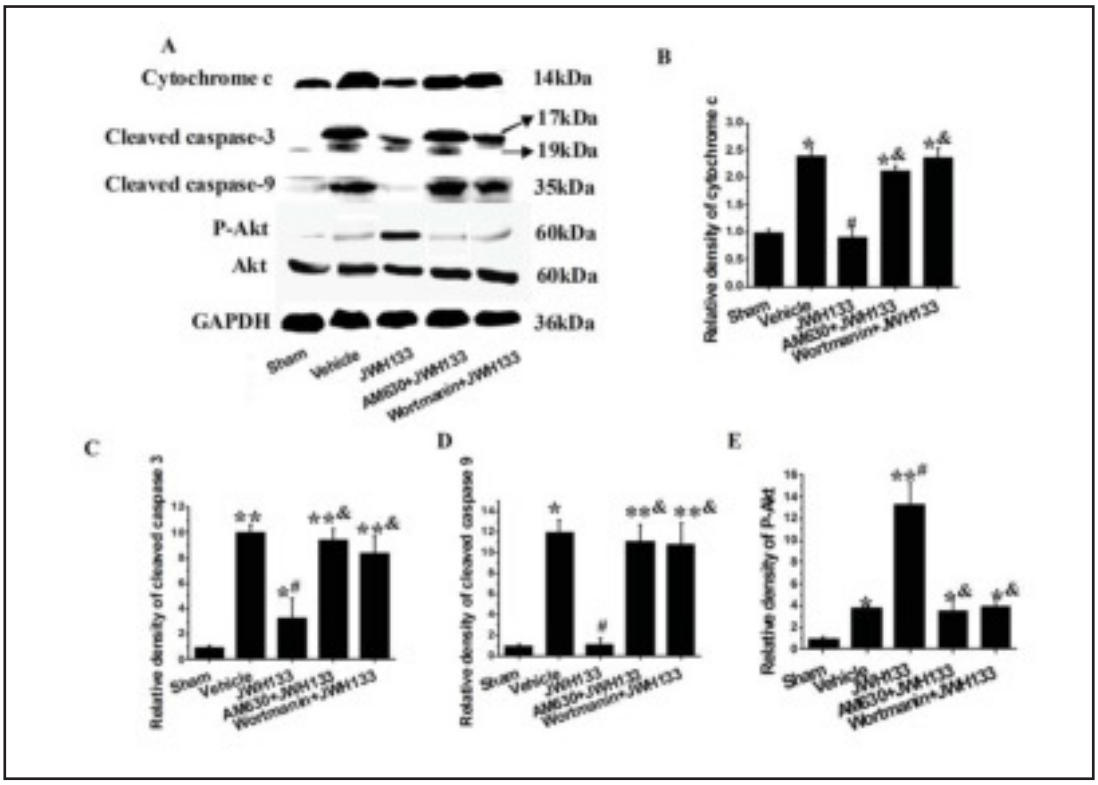

Fig. 4. Western blots analysis of release of mitochondrial cytochrome *c*, cleaved caspase 3, cleaved caspase 9 and P-Akt expression in the myocardium tissues in rats suffering ischemia/reperfusion. (A) Representative western blots of heart lysates (50 μg per lane) of rats from sham group, vehicle treated group, JWH-133 treated group, AM630+JWH133 treated group, and Wortmanin+JWH133 treated group. Bars of relative density of cytochrome c (B), cleaved caspase 3(C), cleaved caspase 9(D) and P-Akt(E). Data were relative density to that in Sham group. n=6 for each group. Data were expressed as mean±SEM.*P<0.05, **P<0.01 vs. Sham group, #P<0.05 vs. vehicle group, &P<0.05 vs. JWH133 group.

anti-apoptosis in myocardium of I/R model. All events induced by I/R, including loss of $\Delta\Psi$m, release of mitochondrial cytochrome *c* to cytosol and activation of caspase 9 and 3, were significantly weakened by JWH133 through activation of Akt. This JWH133-mediated cardioprotective effects were totally abrogated by pre-injection of PI3K inhibitor wortmanin or CB2 receptor antagonist AM630. These results implicated that activation CB2 receptor by JWH133 might render a cardiac protective function via a mitochondrial-dependent pathway, and PI3K/Akt signal pathway might be involved.

Cardiac I/R injury represents a clinically relevant problem associated with thrombolysis, angioplasty, and coronary bypass surgery. For a long time, necrosis was regarded as the sole cause of cell death in myocardial infarction. However, it is recently revealed that apoptosis also plays an important role in the process of cardiomyocyte damage subsequent to myocardial infarction. Many studies have demonstrated that apoptosis is implicated in experimental I/R models [23]. Furthermore, cardiac reperfusion is shown to accelerate the occurrence of apoptotic cell death in cardiomyocytes [24]. Here we showed that cardiomyocytes underwent apoptosis on a large scale during I/R injury. TUNEL data revealed that apoptosis was dramatically increased in hearts subjected to I/R, which was consistent with previous experimental and clinical studies [25, 26]. In present study, administration of JWH133 significantly reduced not only the infarct size but also apoptosis induced by myocardial I/R. AM630, a CB2 receptor antagonist, totally abrogated the effect of JWH133 on infarct size and apoptosis. All these results suggested that JWH133 reduced apoptosis and infarct size through activation of CB2 receptor.

It has been reported that apoptosis can be initiated through the mitochondrial or intrinsic pathway. The role of mitochondria during I/R is particularly critical because of the conditions that promote both apoptosis by the mitochondrial pathway and necrosis by irreversible damage to mitochondria in association with mitochondrial permeability transition. When I/R-induced cellular dysfunctions converge on mitochondrion, mitochondrion undergo massive swelling and become uncoupled as a result of the opening of mitochondrial permeability transition pore (MPTP) [27]. $\Delta\Psi$m is an important parameter of mitochondrial function used as an indicator of cell health. Loss of $\Delta\Psi$m may induce the formation of the MPTP and the subsequent mitochondrial permeability transition [27, 28]. Induction of the MPTP produces a further disruption of the $\Delta\Psi$m and uncoupling of the respiratory chain which promotes the opening of additional MPTP [27]. If MPTP remains open, it will lead to the subsequent release of apoptotic proteins, such as cytochrome *c* and Smac/DIABLO, to play critical roles in apoptosis. The release of apoptotic proteins in turn activates caspase 9, a cysteine protease, and caspases 3 and 7 [29]. Caspase-3, a central 'executioner' or 'downstream' caspase, is an important effector molecule in apoptosis [30], which is responsible for destroying the cell and inducing cell death. Our results showed that I/R induced loss of $\Delta\Psi$m, expression of cleaved caspases-3 and -9, and release of mitochondrial cytochrome *c* to cytosol significantly, that were effectively eliminated by JWH133. Further studies showed that JWH133-mediated effects were totally abrogated by CB2 receptor antagonist AM630. We concluded that JWH133 protected mitochondria against I/R injury and reduced apoptosis through inhibition of apoptotic protein released from MPTP. Furthermore, all of these effects of JWH133 were mediated by CB2 receptor.

The reperfusion injury salvage kinases (RISK), such as Akt and ERK, have been proposed to be linked to the inhibition of the MPTP opening [31]. It has been reported that activation of PI3K/Akt signaling pathway is important for cardioprotection and to inhibit the mitochondrion-mediated pathway of apoptosis in myocardial reperfusion injury [32, 33]. In present study, we showed that JWH133 significantly enhanced Akt phosphorylation and this effect was inhibited by PI3K inhibitor wortmannin and AM630. Our results suggested that JWH133 activated PI3K/Akt signaling pathway through activation CB2 receptor. Similarly, wortmannin could antagonize the protective effects of JWH133 on infarct size, apoptosis, $\Delta\Psi$m loss, and apoptotic proteins levels (cleaved caspase 3 and 9, release of mitochondrial cytochrome *c*) during I/R. Therefore, the PI3K/Akt pathway might serve as a regulator of mitochondrial pathway. when JWH133 protect myocardium against I/R injury. In addition to PI3K/Akt signaling, there may remain other possible mechanisms. More further studies are needed to verify this function.

Apoptosis plays a critical role in tissue damage after myocardial infarction [34]. It was reported that the apoptotic myocytes were most prominent in the border zones of recent infarction, whereas very few apoptotic cells were present in the remote non-infarcted myocardium [35]. It is suggested that apoptosis has some influence on infarct size. In addition to apoptosis, necrosis is a more important determinant of infarct size. Necrosis arises from fatal external insults and results in spillage of the cellular content, with subsequent inflammation [35]. The process of I/R induces mitochondrial dysfunction and MPTP open. These changes will cause irreversible damage to the cell resulting in necrotic death [36]. Furthermore, PI3K/Akt signaling pathway can regulate inflammatory responses and may be an endogenous negative feedback regulator and/or compensatory mechanism that serves to limit pro-inflammatory in response to injurious stimuli. Therefore, activation of PI3K/Akt pathway may reduce necrosis [33]. In present study, JWH133 inhibited the loss of $\Delta\Psi$m and release of mitochondrial cytochrome c to the cytosol during I/R. JWH133 also activated PI3K/Akt signaling pathway. Therefore, we speculated that JWH133 reduced the final infarct size by another mechanism that might be the reduction of necrosis. However, the exactly mechanisms of JWH133 on necrosis need further exploration.

In conclusion, the present study demonstrated for the first time that activation CB2 receptor by JWH133 could inhibit the intrinsic, mitochondria-mediated apoptotic pathway through activation of PI3K/Akt signaling pathway. This was at least the partial mechanisms

through which JWH133 reduced infarct size in rat myocardium suffering I/R injury. It revealed a novel mechanism of cardioprotective action and a potential therapeutic target against I/R injury.

Conflicts of Interest

None.

Acknowledgements

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References

1 Hiley CR: Endocannabinoids and the heart. J Cardiovasc Pharmacol 2009;53:267-276.
2 Steffens S, Pacher P: Targeting cannabinoid receptor cb(2) in cardiovascular disorders: Promises and controversies. Br J Pharmacol 2012;167:313-323.
3 Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI: Structure of a cannabinoid receptor and functional expression of the cloned cdna. Nature 1990;346:561-564.
4 Munro S, Thomas KL, Abu-Shaar M: Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993;365:61-65.
5 Pertwee RG: Pharmacology of cannabinoid cb1 and cb2 receptors. Pharmacol Ther 1997;74:129-180.
6 Pacher P, Hasko G: Endocannabinoids and cannabinoid receptors in ischaemia-reperfusion injury and preconditioning. Br J Pharmacol 2008;153:252-262.
7 Lepicier P, Bibeau-Poirier A, Lagneux C, Servant MJ, Lamontagne D: Signaling pathways involved in the cardioprotective effects of cannabinoids. J Pharmacol Sci 2006;102:155-166.
8 Joyeux M, Arnaud C, Godin-Ribuot D, Demenge P, Lamontagne D, Ribuot C: Endocannabinoids are implicated in the infarct size-reducing effect conferred by heat stress preconditioning in isolated rat hearts. Cardiovasc Res 2002;55:619-625.
9 Lagneux C, Lamontagne D: Involvement of cannabinoids in the cardioprotection induced by lipopolysaccharide. Br J Pharmacol 2001;132:793-796.
10 Lepicier P, Bouchard JF, Lagneux C, Lamontagne D: Endocannabinoids protect the rat isolated heart against ischaemia. Br J Pharmacol 2003;139:805-815.
11 Krylatov AV, Uzhachenko RV, Maslov LN, Ugdyzhekova DS, Bernatskaia NA, Pertwee R, Stefano GB, Makriyannis A: [anandamide and r-(+)-methanandamide prevent development of ischemic and reperfusion arrhythmia in rats by stimulation of cb2-receptors]. Eksp Klin Farmakol 2002;65:6-9.
12 Montecucco F, Lenglet S, Braunersreuther V, Burger F, Pelli G, Bertolotto M, Mach F, Steffens S: Cb(2) cannabinoid receptor activation is cardioprotective in a mouse model of ischemia/reperfusion. J Mol Cell Cardiol 2009;46:612-620.
13 Bialik S, Cryns VL, Drincic A, Miyata S, Wollowick AL, Srinivasan A, Kitsis RN: The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. Circ Res 1999;85:403-414.
14 Black SC, Huang JQ, Rezaiefar P, Radinovic S, Eberhart A, Nicholson DW, Rodger IW: Co-localization of the cysteine protease caspase-3 with apoptotic myocytes after in vivo myocardial ischemia and reperfusion in the rat. J Mol Cell Cardiol 1998;30:733-742.
15 Holly TA, Drincic A, Byun Y, Nakamura S, Harris K, Klocke FJ, Cryns VL: Caspase inhibition reduces myocyte cell death induced by myocardial ischemia and reperfusion in vivo. J Mol Cell Cardiol 1999;31:1709-1715.
16 Mocanu MM, Baxter GF, Yellon DM: Caspase inhibition and limitation of myocardial infarct size: Protection against lethal reperfusion injury. Br J Pharmacol 2000;130:197-200.

17 Defer N, Wan J, Souktani R, Escoubet B, Perier M, Caramelle P, Manin S, Deveaux V, Bourin MC, Zimmer A, Lotersztajn S, Pecker F, Pavoine C: The cannabinoid receptor type 2 promotes cardiac myocyte and fibroblast survival and protects against ischemia/reperfusion-induced cardiomyopathy. FASEB J 2009;23:2120-2130.
18 Borutaite V, Brown GC: Mitochondria in apoptosis of ischemic heart. FEBS Lett 2003;541:1-5.
19 Johnston KM, MacLeod BA, Walker MJ: Responses to ligation of a coronary artery in conscious rats and the actions of antiarrhythmics. Can J Physiol Pharmacol 1983;61:1340-1353.
20 Li Q, Shi M, Li B: Anandamide enhances expression of heat shock protein 72 to protect against ischemia-reperfusion injury in rat heart. J Physiol Sci 2013;63:47-53.
21 Sun CK, Zhang XY, Sheard PW, Mabuchi A, Wheatley AM: Change in mitochondrial membrane potential is the key mechanism in early warm hepatic ischemia-reperfusion injury. Microvasc Res 2005;70:102-110.
22 Jassem W, Fuggle SV, Rela M, Koo DD, Heaton ND: The role of mitochondria in ischemia/reperfusion injury. Transplantation 2002;73:493-499.
23 Fliss H, Gattinger D: Apoptosis in ischemic and reperfused rat myocardium. Circ Res 1996;79:949-956.
24 Haunstetter A, Izumo S: Apoptosis: Basic mechanisms and implications for cardiovascular disease. Circ Res 1998;82:1111-1129.
25 Prech M, Marszalek A, Schroder J, Filas V, Lesiak M, Jemielity M, Araszkiewicz A, Grajek S: Apoptosis as a mechanism for the elimination of cardiomyocytes after acute myocardial infarction. Am J Cardiol 2010;105:1240-1245.
26 Li Y, Ge X, Liu X: The cardioprotective effect of postconditioning is mediated by arc through inhibiting mitochondrial apoptotic pathway. Apoptosis 2009;14:164-172.
27 Baines CP: The mitochondrial permeability transition pore and ischemia-reperfusion injury. Basic Res Cardiol 2009;104:181-188.
28 Halestrap AP, Clarke SJ, Javadov SA: Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res 2004;61:372-385.
29 Gustafsson AB, Gottlieb RA: Heart mitochondria: Gates of life and death. Cardiovasc Res 2008;77:334-343.
30 Uchiyama T, Otani H, Okada T, Ninomiya H, Kido M, Imamura H, Nogi S, Kobayashi Y: Nitric oxide induces caspase-dependent apoptosis and necrosis in neonatal rat cardiomyocytes. J Mol Cell Cardiol 2002;34:1049-1061.
31 Hausenloy DJ, Yellon DM: New directions for protecting the heart against ischaemia-reperfusion injury: Targeting the reperfusion injury salvage kinase (risk)-pathway. Cardiovasc Res 2004;61:448-460.
32 Armstrong SC: Protein kinase activation and myocardial ischemia/reperfusion injury. Cardiovasc Res 2004;61:427-436.
33 Zhu M, Feng J, Lucchinetti E, Fischer G, Xu L, Pedrazzini T, Schaub MC, Zaugg M: Ischemic postconditioning protects remodeled myocardium via the pi3k-pkb/akt reperfusion injury salvage kinase pathway. Cardiovasc Res 2006;72:152-162.
34 Krijnen PA, Nijmeijer R, Meijer CJ, Visser CA, Hack CE, Niessen HW: Apoptosis in myocardial ischaemia and infarction. J Clin Pathol 2002;55:801-811.
35 Scarabelli TM, Gottlieb RA: Functional and clinical repercussions of myocyte apoptosis in the multifaceted damage by ischemia/reperfusion injury: Old and new concepts after 10 years of contributions. Cell Death Differ 2004;11:S144-152.
36 Halestrap AP, Clarke SJ, Khaliulin I: The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta 2007;1767:1007-1031.