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Modulation of Angiotensin Receptors AT1 And AT2 And the Cannabidiol-Mediated RISK PI3K / AKT And MAPK / ERK (CBD) Pathways Reduces Myocardial Damage by Reperfusion and Improves Hemodynamic Variables in Isolated Hearts

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ABSTRACT

Introduction: Myocardial ischemia continues to be the first cause of morbimortality in the world, the definitive treatment is reperfusion, however this action causes additional damage to ischemic myocardial tissue, this forces to seek therapies of cardioprotection to reduce this additional damage. There are many cardioprotective agents, cannabinoids have shown to have important cardioprotective effects mainly cannabidiol (CBD), a non-psychoactive cannabinoid, CB2 receptor agonist.

Objective: To evaluate the effect of Cannabidiol (CBD) on a model of myocardial ischemia-reperfusion in rats.

Material and Methods: 12-week old male Wistar rats, 3 groups, Control(C), Ischemia-reperfusion (IR), CBD pretreatment 10 days dose of 5mg/kg c/24hrs prior to the ischemic-reperfusion (CBD) event. Langendorff organ isolate studies were performed and the area of infarction was assessed with triphenyl tetrazolium, in addition to molecular analysis of AT1, AT2 receptors and Akt, Erk proteins and their phosphorylated forms related to RISK pathways.

Results: It was observed that there is an improvement with the use of CBD increasing inotropism and cardiac lusitropism, improving considerably the cardiovascular functionality. These could be related to the reduction of the area of infarction and activation of the AT2 receptor and the RISK pathway with absence of activation of the AT2 receptor.

Conclusion: The use of cannabinoids was shown to have beneficial effects when used as a treatment for myocardial reperfusion damage.

Introduction

Despite the scientific progress that has been made in the cardiovascular area, myocardial ischemic disease remains as the leading cause of morbidity and mortality worldwide [1], the worldwide established treatment for this condition is the prompt restoration of coronary blood flow. Paradoxically, these procedures generate additional damage that has been well

established as reperfusion damage, causing necrosis and apoptosis of the myocardial cells that are in the area at risk of the ischemic zone, in this zone there is an abundant inflammatory process, oxidative, stress, as well as areas of fibrosis [2,3]. In addition, immediately after myocardial ischemia, the angiotensin renin system is triggered, and AT1 receptor stimulation is responsible for vasoconstriction, sympathetic activation, cell growth (myocardial hypertrophy), and remodeling mediated by fibroblast proliferation and collagen production [4-6]. While it has been studied that the effects of the AT2 receptor are counter-regulatory [7]. We are currently in need of continuing to evaluate molecules with potential cardioprotective effects. Cannabinoids have aroused interest in their therapeutic uses and in evaluating their potential cardioprotective effects. Cannabinoids are a group of compounds of endogenous (endocannabinoids), natural (Phyto cannabinoids) or synthetic origin with structure and pharmacology related to the Cannabis sativa plant, all of which have in common the activation of the CB1 and CB2 receptors [8].

There are more than 70 known cannabinoid derivatives, but the most abundant are two, tetrahydrocannabinol (Δ9-THC) with important psychoactive effects, activating CB1 receptors which are widely distributed over the central nervous system and (-)-cannabidiol (CBD) is a non-psychoactive that has reported that even low doses are an important CB1 receptor antagonist AND CB2 receptor agonist which is distributed mainly on myocardial cells and immune cells [9,15]. The CB2 receptor has been attributed important cardioprotective effects in addition to anti-inflammatory drugs [16] and therefore the effects of reducing damage by restoring coronary flow have been evaluated in multiple studies, both during ischemia and prior to reperfusion [17-21]. Another interesting finding is that CBD can modulate the activation of other receptors such as the Adenosine A1 receptor, generating electrocardiographic and molecular evidence of important myocardial protection [22-24]. On the other hand, for the last 30 years in the field of cardioprotection a molecular pathway that has an important effect on reperfusion damage has been extensively studied. This pathway is the Reperfusion Injury Rescue Kinases (RISK) pathway, which in turn has been linked to the modulation of G-protein-coupled receptors in the heart [25]. Nonetheless , the use of CBD as a cardioprotective agent requires more observation points and it is of our interest to evaluate if the CBD which is administered to animals during the days prior to an ischemia-reperfusion event can generate cardioprotection against a global ischemia-reperfusion event, as well as if this cannabinoid can modulate the activity of the receptors to angiotensin AT1 and AT2, as well as the possible intervention of the RISK pathway in its cardioprotective effects against reperfusion damage. These effects have not been evaluated before.

Material and Methods

AnimalsTwelve-week old male Wistar rats which were provided by the biotherium of the Escuela Superior de Medicina (Instituto Politécnico Nacional, Mexico City), were kept individually in acrylic cages and randomly distributed in 3 different groups (n =6) for functional, morphological and molecular studies:

a) SHAM animals (simulated ischemia-reperfusion surgery) (C),

b) animals with ischemia-reperfusion damage (IR),

c) animals with ischemia-reperfusion damage treated with cannabidiol (CBD DROPYDIOL) 5mg/Kg every 24 hours for 10 days prior to the surgical event injected by intraperitoneal route (IP) The animals were acclimated to laboratory conditions, including a 12 h (12:12) light/dark cycle, for at least seven days. Sterile feed and water were provided ad libitum. The handling of the animals was in accordance with the Mexican Federal Regulations for the Experimentation and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico City, Mexico) and approved by the Institutional Committee for the Care and Use of Animals (CICUAL-ESM-IPN).

Surgical Procedures

The animals were anaesthetized by IP injection of ketamine (100 mg/kg; S.A.G.A.R.P.A. Q-7833-028) and xylazine (10 mg/kg, S.A.G.A.R.P.A. Q-7833-099), intubated and ventilated with positive pressure on a small animal respirator (Harvard). A left thoracotomy was performed to expose the heart. In IR animals, the left anterior descending coronary artery was ligated with a 5Fr nylon suture and a 5Fr feeding catheter was placed over it, for 45 minutes, then the artery was released by removing the feeding catheter and the suture was left in place as a reference point. Successful occlusion and reperfusion were verified by visual inspection of LV color. The thorax was closed in layers and the animals were extubated, lost body fluids were replaced by subcutaneous administration of 30 ml of saline, and the animals were treated with tramadol analgesia (5 mg/kg ip) for 2 days. The simulated animals underwent the surgical procedure described with the exception of coronary ligation and ischemia Collection of tissues and determination of IM size for morphological analysis (Triphenyl Tetrazolium Staining). 48 hours after the surgical procedures the animals were anaesthetized with sodium pentobarbital at a dose of 100mg/Kg and were killed by decapitation. The hearts were then removed and weighed. The area at risk (AAR) was determined by the reocclusion of the anterior descending artery using the suture placed during the surgical procedure and the infusion of 0.5-1 ml of 1% Evans blue solution in saline was slowly infused in a retrograde fashion through the cannulated aorta into the coronary arteries. The heart was then

frozen (-80 °C) for 15 minutes in aluminum foil and 2mm cross sections were made.

Four middle LV rings were taken considering a ratio of the free wall of the left ventricle equivalent to the radius of the ventricular cavity to ensure that all selected rings were taken at the same point and stained with 1% (w/v) triphenyl tetrazolium chloride (TTC) with incubation at 37°C for 15 min in phosphate buffer (pH 7.4) and fixed at 10% (w/v) in formaldehyde solution. The image analysis was performed in Image Pro Plus software. The infarct area (IA) and the area at risk (AAR) were measured. The results are expressed as IA / AAR. In a subgroup of animals from groups 1, 2 and 3, the hearts were removed and prepared with Krebs solution for ex vivo hemodynamic analysis. In another subgroup of animals from groups 1, 2, 3 and 4, the hearts were removed and immediately frozen in liquid nitrogen and stored at -80°C for further molecular analysis. Images of unfixed stained rings were also used to measure internal and external chamber diameters, and anterior and septal wall thicknesses.

Hemodynamic Studies (Langendorff Heart Isolated *Ex Vivo*)

The rats were given heparin (100 U.kg-1, s.c.); after 15 minutes they were anesthetized with sodium pentobarbital (50 mg.kg-1, ip). The animals were slaughtered, their hearts were immediately removed and immersed in Krebs- Henseleit solution (KHS). The hearts were perfused in a retrograde fashion, using an aortic cannula, with a constant flow rate (10ml.min-1). Perfusion was performed using KHS containing (mM): NaCl (118), KCl (4.7), CaCl2 (1.5), $MgSO_4$ (1.2), $NaHCO_3$ (25), $KH2PO_4$ (1.2), dextrose (11) and double-distilled water, balanced with continuous gassing (95% O2 and 5% CO2 at 37°C) to maintain a pH of 7.4. After a 30-minute stabilization period, cardiac function was determined using an isovolumetric technique by Langendorff [17]. Left ventricular pressure was quantified by means of a balloon constructed of plastic film (3-5 mm in diameter) inserted into the left ventricle via the left atrium and connected to a pressure transducer (TSD104A, Biopac Systems Inc., Santa Barbara, CA, USA) coupled to a software (Acknowledge program; MP 100WSW, Biopac Systems Inc.) for data acquisition. The balloon volume was adjusted to produce a pressure at the end of the diastole of 8 to 10 mmHg in all groups [18]. Measurements of left ventricular pressure development (LVDP), maximum value of the first derivative of positive ventricular pressure (+dP/dtmax), minimum negative (- dp/dtmin) and heart rate (HR) were made. Coronary perfusion pressure (CPP) was measured through a lateral connection on the perfusion cannula, connected to a perfusion transducer (TSD104A, Biopac Systems Inc.). Once the baseline values of the cardiac mechanics were obtained, the isolated hearts obtained from animals in each experimental group were subjected to a period of 0 flow global ischemia, completely obstructing the retrograde perfusion for 30 minutes. Subsequently, the isolated hearts were re-infused with KHS for 90 minutes; measurements were taken at 15, 30, 45, 60 and 90 minutes [26-30].

Molecular Analysis (Western Blot)

Table 1: List of antibodies used in Western Blot molecular analysis, brands and concentrations.

Primary antibodies	Brand	Concentration
AT1 Anti-rabbit	Pro-sci 5391	1:2000
AT2 Anti-rabbit	Pro-sci 5393	1:2000
Akt Anti-mouse	sc-81434	1:2500
pAkt Anti-mouse	sc-377556	1:2500
Erk 1/2 Anti-rabbit	sc-153	1:3000
pERK1/2 Anti-rabbit	sc-7383	1:3000
B actina Anti-Goat	sc-1615	1:3000
secondary antibodies (HRP-conjugated)		
Anti-Rabbit (Invitrogen)	656120	1:6000
Anti-Goat (Invitrogen)	81-1620	1:12500
Anti-Mouse (Santa Cruz)	sc-2005	1:8000

100 mg of previously collected heart tissue were taken and kept frozen at -80°C. They were homogenized in polytron in Tris solution pH 7.4 with protease and phosphatase inhibitors (miniComplete Cocktail). They were centrifuged at 4°C at 10,000 G for 15 minutes and protein quantification was performed by Bradford technique. Western Blot was used to determine the variations in AT1R, AT2R, phosphorylated ERK AKT and their relationship with their total forms. Subsequently, 100 µg of total protein were taken from each sample and separated by 10% SDS- PAGE gels under reducing conditions. They were then transferred to a polyvinylidene dichloride membrane. (Immobilon PVDF, 0.45 µm; Millipore, USA). The membranes were then blocked with 5% bovine serum albumin in TBS - 0.1% Tween 20 (TBS-T, pH 7.4) for 2 hours, washed three times with TBS-T, and incubated with primary antibodies listed in (Table 1) at 4°C overnight on continuous agitation. After proper washing with TBS-T, the membranes were incubated with secondary antibodies listed in (Table 1), at room temperature for 2 hours on continuous agitation. Detection was carried out using the enhanced chemiluminescence method (Western transfer luminol reagent, Santa Cruz Cat. 2048). The membranes were photographed, and the image digitized for densitometric analysis using the Image Studio Lite software (LI-COR Biosciences). The relative presence of each protein was normalized with β-actin as the housekeeping protein Statistical analysis. The data are presented as the mean ± SEM. A comparison between 3 groups in the respective data was analyzed, using the one-way ANOVA test with its subsequent posthoc Tukey test considering significant data with a $p<0.05$. All analyses and graphs were performed with Prisma software version 8.0.

Results

Evaluation of basal heart function and its functional response to ischemic reperfusion in untreated and CBD-treated animals. Basal heart function was evaluated by measuring LVDP (mmHg), heart rate (HR, bpm) and LVDP + dP/dtmax and -dP/dtmin, (mmHg/s) and CPP (mmHg). During the stabilization period, they represent an average of the last 5 min of a total 30 minutes of stabilization prior to the total ischemia event, by cutting the continuous flow of solution and carbogen administered by the Langendorff system. The hearts of rats treated with CBD showed a better and faster recovery being significantly higher from the first 15 minutes and persisting until 60 minutes where the total reperfusion period was completed (Table 2). Notwithstanding, the group treated with CBD showed significantly increased recovery with increased cardiac inotropism: with elevated LVDP and +dP/dtmáx(%) compared to the IR group as well and increased lusitropy with increased LVDP and increased -dP/dtmáx(%) . Also, the CPP was significantly increased from the first 15 minutes in the CBD group and remained so until the end of the experiment (Figure 1). This establishes an increase in the force of contraction and the speed of relaxation as well as an increase in the available coronary flow. However, the heart rate showed no change in both IR and CBD groups, either during the basal period of stabilization or after reperfusion and continued unchanged until the end of the experiment, suggesting that no harm was done to cardiac automatism (Figure 2).

Table 2: Recovery percentages at 15 minutes, 30 minutes and 45 minutes after restoring flow and carbogen (reperfusion) in untreated hearts (IR) and in hearts treated 10 days before with CBD in doses of 5mg/Kg, c/24 hrs IP. Means ± SEM are shown considering a n=6 rats per group, *p=<0.005 CBD vs IR.

Post Reperfustion Time(Dp/Dt+)	IR			CBD		
	%Recovery	SEM	n	%Recovery	SEM	n
15min	**30.68**	**5.75**	**6**	**58.12***	**10.23**	**6**
30min	50.14	5.68	6	82.43*	6.16	6
45min	60.63	6.25	6	93.79*	3.63	6
Post Reperfustion Time(Dp/Dt-)	**IR**			**CBD**		
	%Recovery	**SEM**	**n**	**%Recovery**	**SEM**	**n**
15min	40.07	6.55	6	65.04*	8.08	6
30min	60.25	4.97	6	86.73*	4.05	6
45min	70	5.52	6	94.97*	3.3	6

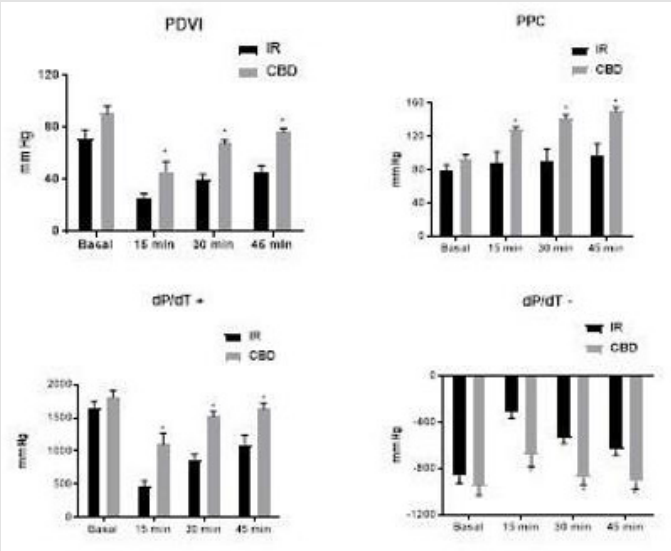

Figure 1: The graphs show

A. left ventricular pressure development (LVDP)

B. coronary perfusion pressure (CPP)

C. maximum value of the first derivative of positive ventricular pressure (+dP/dtmax) and D. first derivative of minimum negative ventricular pressure (-dp/dtmin). From isolated hearts in Langendorff system from untreated (IR) rats and rats treated with CBD dose of 5mg/Kg, c/24hrs, for 10 days prior to the experiment. Baseline measurements were made before reperfusion and at 15 minutes, 30 minutes and 45 minutes after reperfusion, concluding the experiment at 60 minutes after reperfusion. *p<0.05 CBD vs IR.

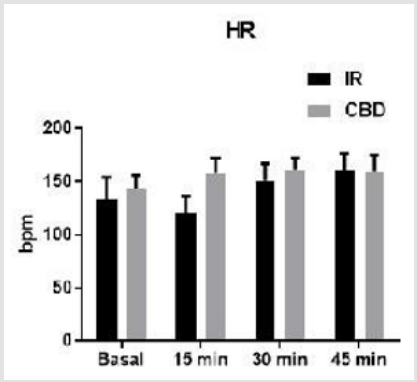

Figure 2: The heart rate (bpm) of the isolated hearts of untreated rats and rats treated with CBD doses of 5mg/Kg, c/24hrs IP, was observed for 10 days prior to the experiment, placed in the Langendorff system. Basal responses were recorded prior to reperfusion and at 15 minutes, 30 minutes and 45 minutes after reperfusion, concluding the experiment at 60 minutes. No significant differences in this variable were found in any temporality.

After the first functional evaluation, confirming that there were effects with the use of CBD, we performed the surgical procedures of anterior descending artery ligation with 45-minute regional ischemia and subsequent reperfusion and were stained with triphenyl tetrazolium stain to assess the RAA(%) and AI/LV(%). There was a statistically significant reduction in the area of IA/LV ischemia (20.55%) and a recovery of healthy tissue of 47.67%. Comparing hearts treated with CBD at a dose of 5mg/Kg, c/24hrs IP, for 10 days prior to the surgical procedure compared to those that received no treatment but underwent ischemic surgery 45 minutes and reperfusion (IR). There was no significant change in the area at risk (AAR) (Figure 3). Finally we evaluated the molecular effect in the left ventricles of the animals submitted to the surgery simulation (SHAM) that will be the control group (C), animals submitted to regional ischemia for 45 minutes with subsequent re-fusion (IR) and the animals with the CBD treatment (CBD) in doses of 5mg/kg, c/24 hrs IP, during 10 days previous to the procedure. Figure 4 shows the differences in the molecular expression of the AT1 and AT2 receptors. It shows a significant increase in the expression of the AT1 receptor in the IR animals without generating elevation of this receptor in the CBD-treated animals (p= 0.0306) or the C group (p= 0.0470) and a significant increase of the AT2 receptor in the CBD-treated group compared to the IR group (p= 0.0002) but also exceeding the basal levels of the AT2 receptor in the control group (p= 0.0005). Figure 4 shows the differences in the molecular expression of the AT1 and AT2 receptors. It shows a significant increase in the expression of the AT1 receptor in the IR animals without generating elevation of this receptor in the CBD-treated animals (p= 0.0306) or the C group (p= 0.0470) and a significant increase of the AT2 receptor in the CBD-treated group compared to the IR group (p= 0.0002) but also exceeding the basal levels of the AT2 receptor in the control group (p= 0.0005).

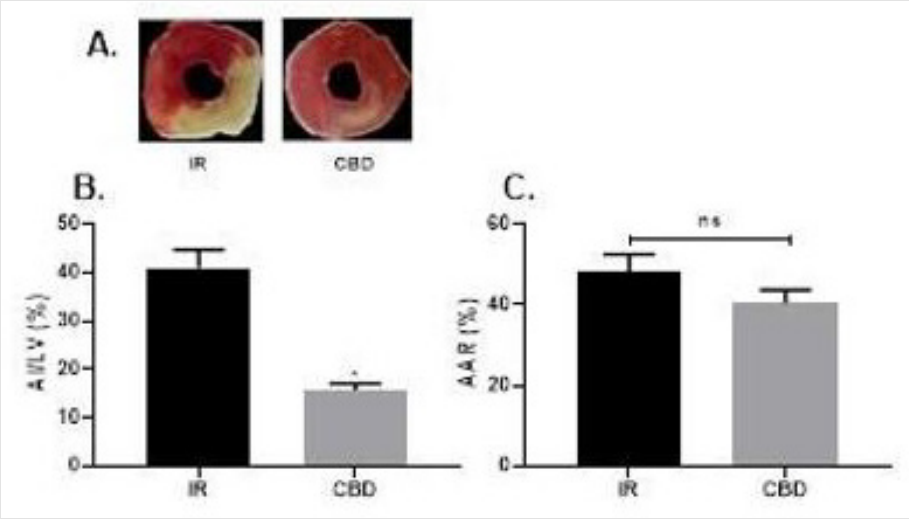

Figure 3: (A) Representative sections of the ring cross section of the left ventricle (LV) animals with ischemia 45 minutes and reperfusion and animals treated with CBD stained with triphenyltetrazolium chloride, (B) Graph of the area of infarct/left ventricle (IA/LV) in coronary occlusion groups for 45 minutes with subsequent reperfusion (n=6). The values are the means ± SEM. *p< 0.001 CBD vs IR.

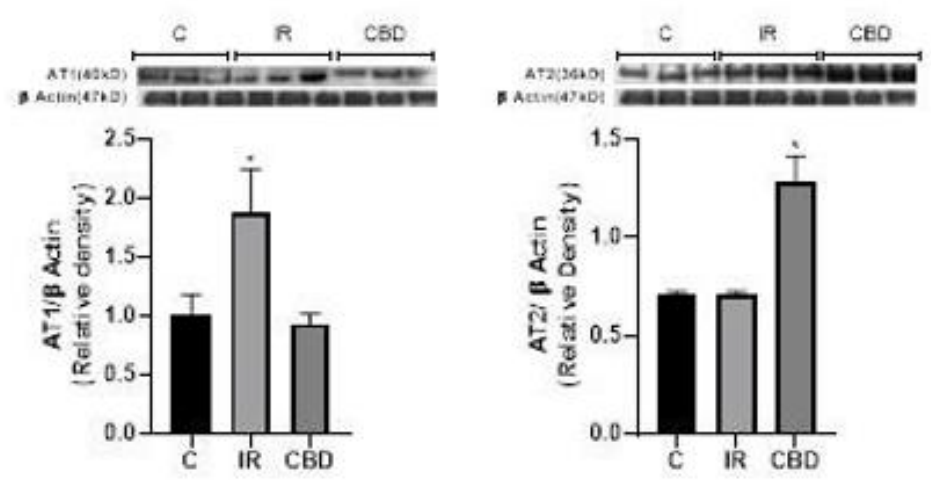

Figure 4: Western blot analysis (A) for AT1 receptor (40 kDa) and (B) for AT2 receptor (36 kDa), in left ventricle of rats submitted to surgery simulation (C), ischemia surgery for 45 minutes and subsequent reperfusion and those treated with CBD doses of 5mg/Kg c/24hrs IP, 10 days prior to the ischemia-reperfusion procedure. A representative Western blot is shown above each graph. The intensity of the bands was quantified by densitometric analysis and normalized with the corresponding β-Actin (47 kDa). The values are averages ± SEM (n = 4) (A)*p < 0.05 IR vs C and CBD; (B) *p<0.05 CBD vs C and IR.

The activation of the RISK pathways characterized by the modulation of the Akt/PKB and ERK proteins and their phosphorylated forms was evaluated. A significant increase in the total forms of AKT was observed in the group treated with CBD compared to group C (p= <0.0001) and also a significant decrease of this protein in the IR animals (p= <0.0001).This decrease is also different from the baseline values of group C (p= 0.0164). As for the phosphorylated forms, we also found a significant increase in the p-Akt levels of the group treated with CBD compared to IR (p= 0.0013) and to group C (p= 0.0003). ERK protein levels were also increased in both total and phosphorylated forms in CBD-treated animals compared to IR-treated and control animals. In general terms we can consider that there is an activation of the RISK pathway at this level (Figure 5).

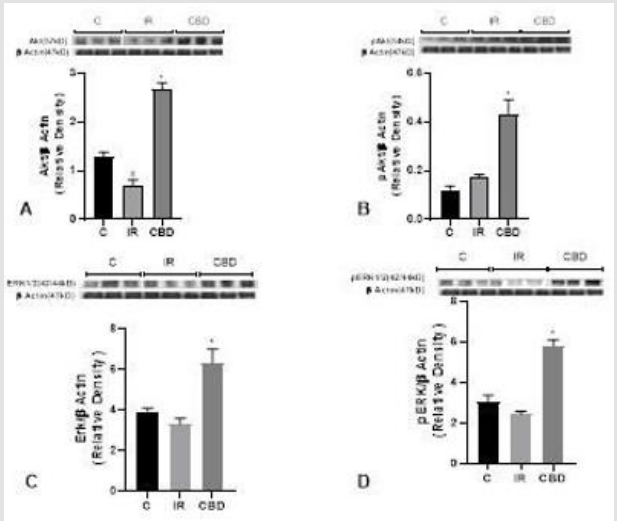

Figure 5: Western blot analysis

A. for the Akt/PKB protein (57 kDa) and

B. its phosphorylated p-Akt form (54 kDa)

C. densitometry of the MAPK/ERK1/2 protein (42/44kDa)

D. its phosphorylated p-ERK1/2 form (44/42 kDa), in the left ventricle of rats subjected to surgical simulation (C), ischemia surgery for 45 minutes and subsequent reperfusion (IR) and those treated with CBD doses of 5mg/Kg c/24hrs IP, 10 days prior to the ischemia-reperfusion procedure. A representative Western blot is shown above each graph. The intensity of the bands was quantified by densitometric analysis and normalized with the corresponding β-Actin (47 kDa). The values are averages ± SEM (n = 4). (A)*p < 0.05 CBD vs IR and C and #p <0.05 IR vs C and CBD; (B) *p<0.05 CBD vs C and IR: (C, D)*vs C and IR.

Discussion

Our findings in ex vivo hearts mounted in the Langendorff system, allowed us to establish that CBD treatment administered prior to an ischemia-reperfusion event can produce a cardioprotective effect that is perpetuated in the myocardial tissue and that is importantly dependent on functional changes at the myocardial level. It is important to note that although no functional change occurs at the baseline, myocardial tissue subjected to reperfusion injury has a higher percentage of recovery of function and this occurs more rapidly within 15 minutes (Figure 1). This is mentioned by some authors who used CBD during the ischemic event and prior to reperfusion, interestingly previous administration of CBD on an ongoing basis exerts similar effects [31-35]. On the other hand, results in the in vivo hearts confirmed that CBD used prior to an ischemia-reperfusion event has a significant cardioprotective effect against additional damage induced by anterior descending artery ligation, with reduction in infarct size measured as a percentage of the area at risk for TTC staining (Figure 2). This was accompanied by an increase in left ventricular function for inotropism and lusitropism, (Table 1) [36-40].

Reduced stroke size was associated with increased expression of the AT2 receptor but no elevation of the AT1 receptor, which has been described as cardioprotective by several authors [41-45]. It has been mentioned that in an acute ischemic event, the AT1 receptor is immediately activated and modulates vasoconstriction, leukocyte infiltration, inflammation and cardiac remodeling actions, which will end up decreasing the physiological capacities of the heart. However, what we find when CBD is administered is an increase in AT2 that can be related to the increase of ventricular functions in the isolated heart (Table 1 and Figure 3). By relating these data to the increase in the AT2 receptor which has been linked to the generation of cardioprotection by having counter-regulatory effects on the AT1 receptor, helping to establish a strong functional relationship of cannabinoids and the modulation of the angiotensin renin system [46-50].

Finally, it was allowed to link the cardioprotective action of CBD to the activation of the RISK pathway [51], which has long been described to exert intrinsic cellular regulatory effects on the protection of myocardial cells from reperfusion injury of the RISK pathways headed by the activation of the PI3K/Akt pathway and the MAPK/RISK pathway, it has been determined that when elevated as in the results obtained in (Figure 5), interestingly it has been described that a reduction of these pathways can generate heart failure due to pathological myocardial hypertrophy, with the consequent remodeling [52-57]. So we could consider that its lack of activation has similar consequences to the activation of the AT1 receptor, so by having the results obtained, we can correlate that the activation of the AT2 receptor is somehow linked to the positive modulation of the RISK pathways and that together they produce a cardioprotection and a fast and better recovery of the ventricular capacities besides reducing the size of the damaged area [58-65].

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