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Original Article

Characterization of hypotensive and vasorelaxant effects of PHAR-DBH-Me a new cannabinoid receptor agonist

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ABSTRACT The effect of PHAR-DBH-Me, a cannabinoid receptor agonist, on different cardiovascular responses in adult male rats was analyzed. The blood pressure was measured directly and indirectly. The coronary flow was measured by Langendorff preparation, and vasomotor responses induced by PHAR-DBH-Me in aortic rings pre-contracted with phenylephrine (PHEN) were analyzed. The intravenous injection of the compound PHAR-DBH-Me (0.018–185 μg/kg) resulted in decreased blood pressure; maximum effect was observed at the dose of 1,850 μg/kg. A concentration-dependent increase in the coronary flow was observed in a Langendorff preparation. In the aortic rings, with and without endothelium, pre-contracted with PHEN (10^{-6} M), the addition of PHAR-DBH-Me to the superfusion solution (10^{-12}–10^{-5} M), produced a vasodilator response, which depends on the concentration and presence of the endothelium. L-NAME inhibited these effects. Addition of CB_1 receptor antagonist (AM 251) did not modify the response, while CB_2 receptor antagonist (AM630) decreased the potency of relaxation elicited by PHAR-DBH-Me. Indomethacin shifted the curve concentration-response to the left and produced an increase in the magnitude of the maximum endothelium dependent response to this compound. The maximum effect of PHAR-DBH-Me was observed with the concentration of 10^{-5} M. These results show that PHAR-DBH-Me has a concentration-dependent and endothelium-dependent vasodilator effect through CB_2 receptor. This vasodilation is probably mediated by the synthesis/release of NO. On the other hand, it is suggested that PHAR-DBH-Me also induces the release of a vasoconstrictor prostanoid.

INTRODUCTION

The endogenous cannabinoid system is a ubiquitous lipidic signaling system that appeared early in evolution and which has essential regulatory functions throughout the body in all vertebrates. Among endocannabinoids (endogenous cannabis-like substances), most studied are three molecules derived from arachidonic acid: anandamide (arachidonoylethanolamide)

[1], 2-arachidonoylglycerol [2] and Oleamide (Oleamide [(Z)-9-octadecenamide]) [3]. The endocannabinoid system consists of enzymes responsible for its biosynthesis, cellular uptake, metabolism, and cannabinoid receptors (the best studied, CB_1 and CB_2) coupled to G proteins [4]. This system is a modulator of the physiological processes not only in the nervous system but also in the endocrine network, the immune system, the gastrointestinal tract, the reproductive system, and the cardiovascular

system [5,6]. On the cardiovascular system, endocannabinoids have been shown to play an essential role in both physiological and pathological processes [7]. Animal models have shown that activation of CB_2 receptors (unlike the CB_1 receptors, their activation does not modify the release of neurotransmitters) induces vasodilation in coronary arteries and decreases cardiac output [8]. Furthermore, this activation diminishes the damage caused by myocardial, cerebral, and hepatic ischemia-reperfusion processes, decreases the inflammatory response and activation of endothelial cells, attenuating leukocyte chemotaxis, endothelial adhesion, transendothelial activation, migration, and related oxidative/nitrosative stress [9]. Recent pharmacological advances have led to the synthesis of cannabinoid receptor agonists and antagonists, more potent and selective. These new tools have enabled the study of the physiological roles played by the cannabinoids and have opened new strategies in the treatment of several diseases such as pain, obesity, neurological dysfunctions, some psychiatric and emotional disturbances, and some cardiovascular diseases [10]. Those compounds include newly synthesized (R,Z)-18-((1S,4S)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl)-18-oxooctadec-9-en-7-yl phenylacetate (PHAR-DBH-Me) that binds and activates the CB receptor. In a previous paper, it has been reported that amide (PHAR-DBH-Me), an agonist of the CB_1, CB_2, and TRPV1 receptors, is synthesized from phenylacetylricinoleic acid and (1S,4S)-2,5-diazabicyclo[2.2.1]heptane [11]. The present study aimed to analyze the effects of the compound PHAR-DBH-Me with different experimental approaches on the cardiovascular system.

METHODS

Animals

The experiments were carried out on rats of the Wistar strain, males, weighing 250 to 300 g, from the Bioterium of the Facultad de Medicina, UNAM. All animals were kept in individual cages at controlled room temperature in a 12-h light-dark cycle with food and water ad libitum. The animals used in this work were handled with the requirements published by SAGARPA in the Technical Specifications for the Production, Care and Use of Laboratory Animals (NOM-062-ZOO-1999), and in compliance with international rules as the Guide for the Care and Use of Laboratory Animals (National Research Council). Besides, the experimental procedures applied in this study was carried out according to the regulations stipulated by the Ethics Committee of Facultad de Medicina, UNAM, under license of the project 001/2011.

Drugs

PHAR-DBH-Me was synthesized at Facultad de Estudios Superiores de Zaragoza-UNAM, as described by López-Ortiz and co-workers [11]. All other drugs were purchased from Sigma-Aldrich. Phenylephrine, carbachol, L-NAME, AM251 and AM630 were dissolved in distilled water. The PHAR-DBH-Me was dissolved in Tyrode and 0.01% Tween 20. Indomethacin was dissolved, by sonication, in 4% sodium carbonate solution. All solutions were prepared on the same day of the experiment. The chemical structure of PHAR-DBH-Me is as follows (Fig. 1).

Measurement of blood pressure (BP)

The effect of PHAR-DBH-Me on BP was analyzed with indirect and direct method.

Indirect method

The animals were conditioned for a week to remain immobilized in a chamber and with a cuff placed at the base of the tail for half an hour, always at the same time of day. At the end of this period, animals were anesthetized with chloralose (40 mg/kg) and urethane (1,200 mg/kg) administered intraperitoneally. They underwent a surgical procedure to place a catheter in the jugular vein, which served to dispense PHAR-DBH-Me, drugs, and solutions. The catheter was tunneled subcutaneously to the back behind the ear and through an incision between the scapulae of the animal and attached to the skin by sutures leaving 2.5–3.0 cm exterior to the skin. The catheter was fixed and covered with PVC tubing for protect it. Subsequently, 48 h were left in postsurgical recovery with free access to food and water.

BP measurement was performed with a "tail-cuff" device (LE 5001 Pressure Meter, Panlab; Harvard Apparatus, Barcelona, Spain), which consists of a cuff that is placed on the tail of animals and a sensor which detects the volume pressure in the tail of the rat. The included software allows seeing the data in real-time continuously. The BP measurements were always made at the same time to avoid variations due to the influence of the circadian rhythm. The measurements were obtained after animals remained, half an hour prior to and throughout the measurement cycle, at a temperature of 28–30°C, to promote vasodilation of the tail's blood vessels. This was achieved by placing the animals between two sources of moderate heat. The data was captured and analyzed by a computer setup.

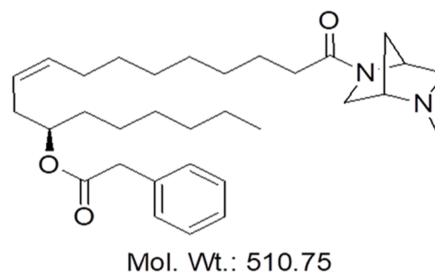Drugs and solutions were administered through the catheter

Fig. 1. Chemical structure of PHAR-DBH-Me (IMM-59). Mol. Wt. 510.75 g/mol.

previously placed in the jugular vein. The control group rats received the volume of the drug administered per dose to observe its effect on blood pressure. Animals from the experimental group received the vehicle plus the experimental compound.

Direct method

Rats anesthetized with chloralose (40 mg/kg) and urethane (1,200 mg/kg) administered intraperitoneally were used. This combination of anesthetics induces an adequate level of anesthesia for prolonged periods, without interfering with cardiovascular function or altering the reflexes that regulate cardiovascular function [12,13]. A tracheostomy was performed previously to ensure adequate ventilation of the animal during the surgery; the animal's body temperature was maintained at 37°C with a thermal pad provided with thermostatic control. Blood pressure was measured utilizing a cannula introduced into the right femoral artery, which was connected in parallel to a mercury manometer and a pressure transducer (model PT-300; Grass Instrument Division, Warwick, NY, USA). The administration of PHAR-DBH-Me, drugs, and solutions were carried out through a cannula placed in the left femoral vein. To avoid the formation of clots inside the cannula, heparin was administered (500–l,000 U/kg i.v.).

In vitro experiments

Determination of total coronary flow in the isolated heart

The Langendorff preparation was utilized to estimate the total coronary flow in the isolated and perfused heart of the rat. For the extraction of the heart, the rat was euthanized by decapitation. Immediately afterward, a thoracotomy was performed, the heart was identified and carefully extracted by cutting the large vessels and trying to preserve the most significant possible portion of the ascending aorta. The heart was immediately fixed to a glass cannula connected to the perfusion system, using 2-0 silk, and perfusion was started in order to avoid any secondary damage to ischemia. The heart, kept in a humid chamber, was perfused continuously with a Tyrode solution aerated with carbogen, at 37°C. The height of the column was maintained at 100 cm in height (constant pressure). The total flow through the coronaries was estimated by measuring every 60 sec the volume of the effluent.

Aortic rings

After 12 h of fasting, the animals were euthanized by decapitation and exsanguination to measure vascular tone. Through a thoracotomy, the thoracic aorta was carefully extracted and placed in a dissection chamber with oxygenated Tyrode solution. Under microscopic observation, it was carefully dissected until it was free of periadventitial connective tissue.

Once dissection was complete, the aorta was cut transversely to obtain 2 mm wide rings. Special care is taken to avoid damage to the endothelium. For each experiment, a pair of rings obtained from the central portion of the same aorta (one with intact endothelium, the other without a functional endothelium) is used. Afterwards in every other ring, the endothelium is removed by

gently rubbing the rings. Each of these rings is suspended horizontally in the same miniature organ chamber (volume 0.5 ml) between two tiny (0.5 mm diameter) stainless steel hooks. One of the hooks is fixed to the chamber wall while the other is attached to an isometric force transducer. The rings were initially stretched until reaching the optimal basal tension (2 g) and were allowed to stabilize for 30 min; during this period the resting tension is constantly monitored and, if needed, readjusted to 2 g by further stretching. The vessels were continuously superfused with pre-warmed (37°C) aerated (95% O_2 and 5% CO_2) modified Tyrode's solution (composition in mM: NaCl, 137; KCl, 2.7; $MgCl_2$, 0.69; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; $CaCl_2$, 1.8 and glucose, 10; pH was adjusted to 7.4). The Tyrode's or test solutions flowed at a rate of 1 ml/min driven by a peristaltic pump. Before entering at the bottom of the miniature organ bath, solutions flow through a spiral immersed in water at a thermostatically controlled temperature. When a solution was changed, the arrival of the new solution at the bottom of the bath could be monitored by the small air bubble preceding its inflow. Solutions were drained by overflow assisted by a cellulose wick to prevent volume change.

Before starting an actual experiment, responsiveness of each pair of rings to the α_1 adrenoceptor agonist phenylephrine and to the stable cholinergic agonist carbachol (carbamoyl choline) was tested. This was achieved by switching the superfusing Tyrode's solution for 10 min to one containing phenylephrine (10^{-5} M) and, thereafter, to one containing, in addition to phenylephrine, carbachol (10^{-5} M). Development of a vigorous (2.0–3.0 g) and sustained contracture in response to phenylephrine evidenced the functional integrity of the smooth muscle layer. Carbachol-induced relaxation of the phenylephrine precontracted vessels were taken as evidence for the preservation of an intact endothelium whereas lack of relaxation confirmed the absence of a functional endothelium [14]. Thus, it was possible to maintain under the same experimental conditions the two rings and record simultaneously the effects on vascular smooth muscle and those where the endothelium was involved.

Experimental protocol

To determinate the mechanism involved in the effect induced by PHAR-DBH-Me on phenylephrine-precontracted rat aortic rings, two main sets of experiments were performed.

First set of experiments

Thirty minutes after restoration of basal tension, 10^{-6} M phenylephrine was added to rat aortic rings with and without endothelium. Ten minutes later, after phenylephrine-induced contraction plateaued, PHAR-DBH-Me began to be cumulatively added (10^{-12}–10^{-5} M) at intervals of around 4–5 min. Tension is reported as a percentage of the phenylephrine-induced contraction.

Second set of experiments

Thirty minutes after adding 10^{-6} M phenylephrine (see first set of experiments), aortic rings with intact endothelium were

preincubated for 30 min with one or two compounds in order to explore the mechanisms involved in the vasorelaxant effect produced by PHAR-DBH-Me. The compounds used for preincubate were: i) 300 μM L-NAME, an inhibitor of NO synthase [14]; ii) 10^{-6} M AM251, a cannabinoid CB_1 receptor antagonist; iii) 10^{-6} M AM630, a cannabinoid CB_2 receptor antagonist [15]; iv) 10^{-6} M indomethacin, a prostaglandin synthesis inhibitor. Subsequently, PHAR-DBH-Me was cumulatively added (10^{-12}–10^{-5} M) at interval around 4–5 min.

Registration system

The tension developed by each ring was recorded utilizing an isometric force transducer (Grass FT03), and the signal was then taken to a model 79 Grass polygraph. The tension of each ring was recorded continuously on paper and was simultaneously digitized (PowerLab 7200; ADI instruments, Soa Paulo, Brasil) and stored on the hard drive of a computer for further analysis.

Data analysis

The variations in vascular tone induced by PHAR-DBH-Me are expressed as the percentage of the maximum tension induced by PHEN (10^{-6} M). pD_2 (–Log of the mean molar concentration of agonist producing 50% of the maximal response) were determined with the software package GraphPad Software Prism 7.0 (San Diego, CA, USA). Comparisons of means were made by two Way Analysis of Variance (ANOVA) and differences among groups were evaluated using Student-Newman-Keuls Method (Sigma Stat software; St. Louis, MO, USA). A p-value of 0.05 or less was considered significant.

RESULTS

Effects of PHAR-DBH-Me on mean blood pressure

To assess the effect of PHAR-DBH-Me in blood pressure, dose-response curves were made using increasing doses of PHAR-DBH-Me (0.018–185 μg/kg). The animals were divided into 2 groups; the control group received only the volume of vehicle used to supply the drugs to check if the volume of the vehicle administered in each dose induces, independently, an effect on the blood pressure, and the experimental group of animals additionally received the compound PHAR-DBH-Me, to evaluate blood pressure by direct and indirect method.

PHAR-DBH-Me produced a decrease in blood pressure measured by direct method, which was significant in comparison with the control group. The maximum decrease in mean arterial pressure was observed with the dose of 185 μg/kg (44.24 ± 8.68 mmHg $vs.$ 103.22 ± 8.98 mmHg, p < 0.05) (Fig. 2A). For the indirect measurement of blood pressure, PHAR-DBH-Me produced a decrease in blood pressure, which was dose-dependent and significant compared to the control group (p < 0.05). The maximum decrease in mean arterial pressure was observed with the concentration of 185 μg/kg (63.17 ± 4.81 mmHg of the experimental group $vs.$ 117.62 ± 2.52 mmHg of the control group, p < 0.05) (Fig. 2B, Table 1). This hypotensive effect was slighter than that observed using the direct method (44.24 ± 8.68 mmHg with the direct method $vs.$ 63.17 ± 9.62 mmHg with the indirect method).

The effect was transient and dose-dependent; the restoration (nearby 90%) of the blood pressure obtained before the administration of PHAR-DBH-Me, was observed after 5 to 10 min, possibly due to the activation of compensation mechanisms at the cardiovascular level.

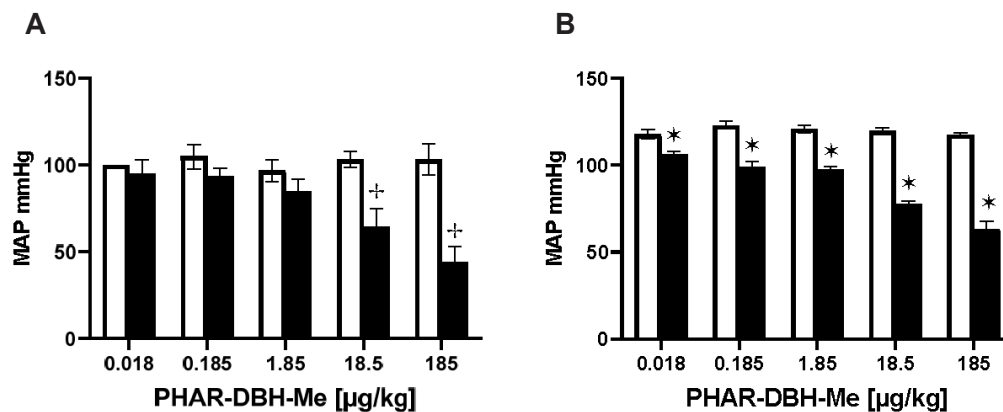**Fig. 2. Effect of PHAR-DBH-Me (0.018–185 μg/kg) on the mean arterial pressure (MAP), determined with the direct (A) and indirect (B) method.** Control (white bars); PHAR-DBH-Me (black bars). The data represents the average of the decrease in blood pressure (mmHg). Data are reported as the mean ± SD of 5 different animals in each group. †p < 0.05 between direct groups and *p < 0.05 between indirect groups.

Table 1. Effect of PHAR-DBH-Me on mean blood pressure measured with two methods

Condition	ED_{50}	MAP (mmHg)
Direct method		
Control (vehicle)	Infinite	103.22 ± 8.98
PHAR-DBH-Me	135.2 µg/kg	44.24 ± 8.68*
Indirect method		
Control (vehicle)	Infinite	117.62 ± 2.52
PHAR-DBH-Me	121.3 µg/kg	63.17 ± 9.62*

Values are presented as mean ± SD. ED_{50}: Dose that produced 50% of the maximum decrease in mean arterial pressure induced by PHAR-DBH-Me (1,850 µg/kg). MAP, mean arterial pressure. *Denotes significant differences between the groups ($p < 0.05$). n = 5 rats in each group.

Effect of PHAR-DBH-Me on coronary flow

The effects of PHAR-DBH-Me on coronary flow (ml/min) were analyzed using the Langendorff preparation. After heart extraction, special care was taken to fix the heart and restore perfusion in the shortest possible time to avoid myocardial ischemia and damage the tissue. The hearts were initially perfused with the Tyrode solution for a period of 10 min. The coronary flow was evaluated every 60 sec to determine their basal value. Subsequently, cumulative concentration-response curves were made using increasing concentrations of PHAR-DBH-Me (10^{-7}–10^{-3} M; concentrations below 10^{-7} M have no significant effect), added to the perfusion solution, and coronary flow was evaluated every 60 sec for 5 min for each dose administered. An increase in coronary flow was produced by PHAR-DBH-Me, which was dependent on the concentration. The maximum increase of flow was observed with the concentration of 10^{-3} M (130.70 ± 7.00%), which was significant in comparison with the coronary flow in basal conditions (Fig. 3).

Role of endothelium and nitric oxide in the effect of PHAR-DBH-Me on aortic rings

Cumulative concentration-response curves were made, adding cumulative concentrations of PHAR-DBH-Me (10^{-13}–10^{-5} M) to the superfusion solution, using rings of aorta pre-contracted with PHEN (10^{-6} M). PHAR-DBH-Me produced a relaxing effect, which was dependent on the concentration and the presence of endothelium. In rings without endothelium, PHAR-DBH-Me had not relaxant effect, instead, a slight increase in tension development was observed. The maximum relaxation was observed with a concentration of 10^{-5} M (66.80 ± 8.12% with endothelium *vs.* 105.33 ± 9.27% without endothelium, $p < 0.05$) and pD_2 for aortic rings with endothelium was 6.12 ± 0.46.

On the other hand, the contribution of nitric oxide to the PHAR-DBH-Me-induced relaxant response was determined adding L-NAME (300 µM) to Tyrode's solution containing PHEN (10^{-6} M). Relaxant differences from control were observed in aor-
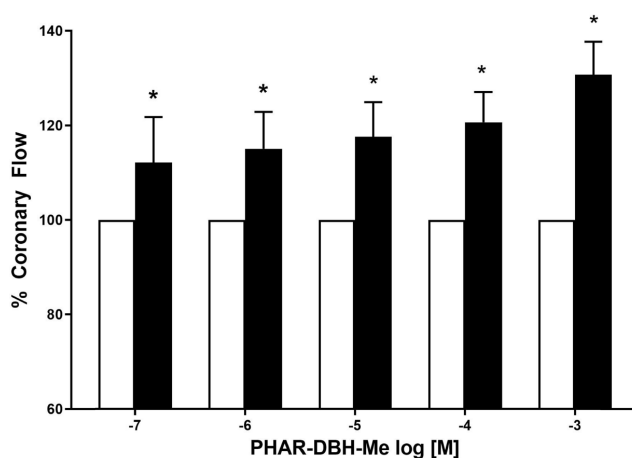

Fig. 3. Effect of PHAR-DBH-Me (10^{-7}–10^{-3} M) on coronary flow with isolated heart preparation. Basal conditions (white bars); treated with PHAR-DBH-Me (black bars). The data represent the average percentage of the increase in coronary flow (ml/min) ± SD of 5 different animals in each group. *$p < 0.05$ *vs.* control (ANOVA followed by modified Newman-Keuls t-test).
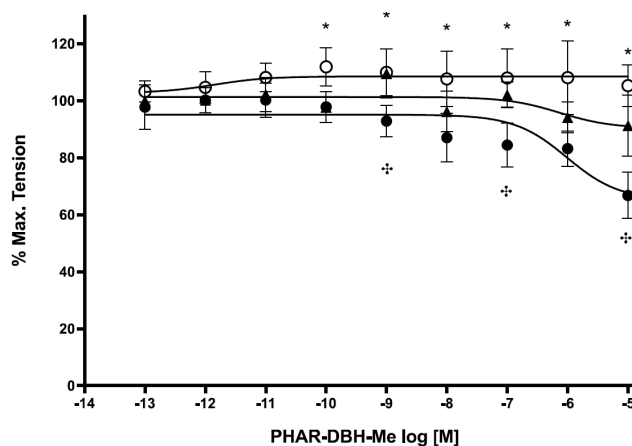

Fig. 4. Concentration-dependent relaxation of 10^{-12}–10^{-5} M PhAR-DBH-Me in 10^{-6} M phenylephrine pre-contracted rat aortic rings with (●) and without (○) endothelium and in presence of L-NAME (300 µM) with endothelium (▲). The data are expressed as the percentage of the maximum tension induced by phenylephrine and are shown as the mean ± SD, n = 5 rats in each group. *Denotes that the differences are significant ($p < 0.05$) between ● and ○. †Denotes that the differences are significant ($p < 0.05$) between ● and ▲.

tic rings with endothelium (91.35 ± 10.75% *vs.* 66.80 ± 8.12%, $p < 0.05$). In aortic rings without endothelium, PHAR-DBH-Me in the presence of L-NAME did not induce a significant relaxation (92.99 ± 5.27%). These results suggest that nitric oxide may be involved at least partially in the PHAR-DBH-Me - induced relaxing response on rat aortic rings (Fig. 4).
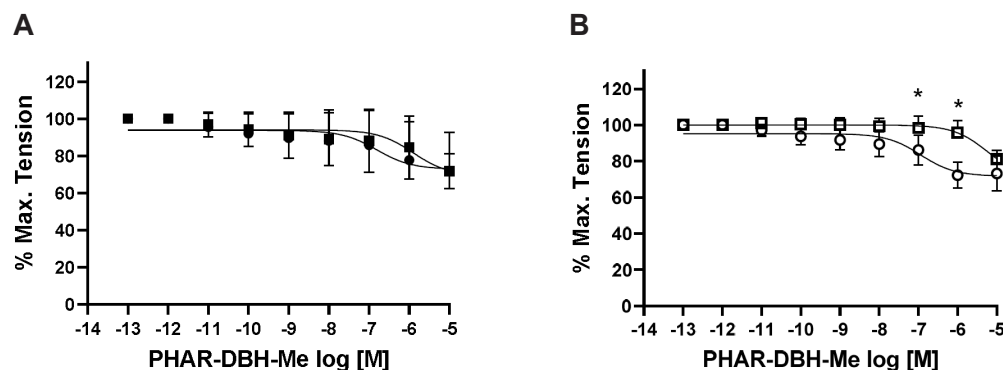
A **B**

Fig. 5. Effect of PHAR-DBH-Me (10^{-12}–10^{-5} M) in aortic rings pre-contracted with phenylephrine (10^{-6} M) in presence of CB_1 receptor antagonist (AM251) (A) and CB_2 receptor antagonist (AM630) (B) (■ and □, respectively). The data are expressed as the percentage of the maximum tension induced by phenylephrine and are shown as the mean ± SD, n = 5 rats in each group. *Denotes that the differences are significant ($p < 0.05$) between ○ and □.

Table 2. Effect of AM251 and AM630 on relaxing effects of PHAR-DBH-Me on aortic rings with endothelium pre-contracted with PHEN

Value	Groups			
	PHAR-DBH-Me (Control 1)	PHAR-DBH-Me + AM251	PHAR-DBH-Me (Control 2)	PHAR-DBH-Me + AM630
pD_2	6.77 ± 0.74	5.88 ± 0.83	6.92 ± 0.69	5.24 ± 0.57*
% Max. Tension	71.56 ± 9.39	71.81 ± 4.24	73.13 ± 9.66	81.35 ± 4.70

Data are presented as the average ± SD. pD_2: –Log of the average of the molar concentration that produced 50% of the maximum relaxation induced by PHAR-DBH-Me (10^{-12}–10^{-5} M). Max. Tension corresponds to the average percentage of the tension decrease compared to the maximum tension developed in response to phenylephrine (PHEN) (10^{-6} M). PHAR-DBH-Me (Control 1): control group of AM251 treatment; PHAR-DBH-Me + AM251: PHAR-DBH-Me in presence of AM251 (10^{-6} M) a antagonist CB_1 receptor; PHAR-DBH-Me (Control 2): control group of AM630 treatment; PHAR-DBH-Me + AM630: PHAR-DBH-Me in presence of AM630 (10^{-6} M) a antagonist CB_2 receptor. *Denotes that the differences are significant respect to the control group ($p < 0.05$). n = 5 rats in each group.

Contribution of cannabinoid receptor CB_1 and CB_2 in the effect of PHAR-DBH-Me

The addition of AM251 (10^{-6} M), antagonist of the CB_1 receptor, did not change the potency of PHAR-DBH-Me or the relaxant response in rings with endothelium pre-contracted with PHEN (71.56 ± 9.39% *vs.* 71.81 ± 4.24%; Fig. 5A). On the other hand, when PHAR-DBH-Me was added to the superfusion solution in the presence of AM630 (10^{-6} M), antagonist of the CB_2 receptor, the concentration-response curve displaced significantly to the right (pD_2 6.92 ± 0.69 *vs.* 5.24 ± 0.57, respectively, $p < 0.05$; Table 2) while the maximum relaxation, slightly decreased respect to the control (73.13 ± 9.66% *vs.* 81.35 ± 4.70%; Fig. 5B). This decrease in PHAR-DBH-Me potency in the presence of AM630 suggests that this compound acts competitively through CB_2 cannabinoid receptors.

Participation of prostanoids on the effect of PHAR-DBH-Me

In the presence of indomethacin (10^{-6} M), a non-selective inhibitor of the cyclooxygenase pathway, PHAR-DBH-Me caused a significant increase in the relaxant response in rings with endothelium pre-contracted with PHEN (66.80 ± 8.12% *vs.* 44.76 ± 8.75%, respectively; Fig. 6A). The concentration-response curve was displaced to the left (pD_2 6.01 ± 0.46 *vs.* 9.48 ± 0.54, respectively, $p < 0.05$; Table 3). On the other hand, when PHAR-DBH-Me was added to the superfusion solution in the presence of L-NAME (300 μM) and indomethacin (10^{-6} M), it was observed a major relaxant response respect in the presence of only L-NAME (Fig. 6B). The finding that indomethacin increases the relaxing response to PHAR-DBH-Me in rings with endothelium suggests that PHAR-DBH-Me induces the release of some vasoconstrictor prostanoid, whose synthesis/release is blocked by indomethacin.

DISCUSSION

In this study, it was demonstrated that the compound PHAR-DBH-Me generates cardiovascular effects causing hypotension in the in vivo studies, as well as relaxation in the blood vessels in the in vitro studies. These effects seem to be related to the presence of endothelium, nitric oxide synthesis and cannabinoid CB_2 receptor.

PHAR-DBH-Me produces a severe hypotension effect, which is greater when the BP measurement is made by the direct method
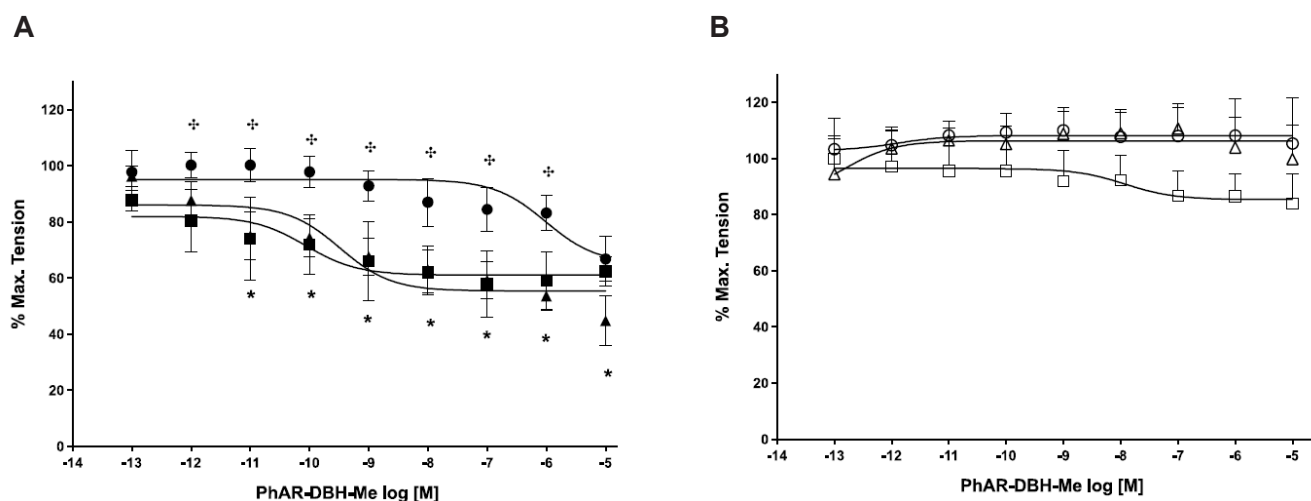
A

B

Fig. 6. Effect of indomethacin (10^{-6} M) on the cumulative concentration-response curve to PHAR-DBH-Me (10^{-12}–10^{-5} M) in aortic rings with (A) and without endothelium (B) pre-contracted with phenylephrine (10^{-6} M) (● and ○), in presence of indomethacin (▲ and △) and L-NAME (300 μM) plus indomethacin (■ and □). The data are expressed as the percentage of the maximum tension induced by phenylephrine and are shown as the mean ± SD, n = 5 rat in each group. *Denotes that the differences are significant ($p < 0.05$) between ● and ▲. [†]Denotes that the differences are significant ($p < 0.05$) between ● and ■.

Table 3. Effect of L-NAME and indomethacin on relaxing effects of PHAR-DBH-Me on aortic rings with and without endothelium pre-contracted with PHEN

Condition	Groups			
	PHAR-DBH-Me	PHAR-DBH-Me + Indo	PHAR-DBH-Me + L-NAME	PHAR-DBH-Me + Indo + L-NAME
With endothelium				
pD$_2$	6.01 ± 0.46	9.48 ± 0.54*	6.13 ± 0.92*	10.06 ± 0.36*
% Max. Tension	66.80 ± 8.12	44.76 ± 8.75*	91.35 ± 10.75*	62.35 ± 5.34
Without endothelium				
pD$_2$	Infinite	Infinite	7.64 ± 0.57*	7.92 ± 0.39
% Max. Tension	105.33 ± 9.27	99.74 ± 12.07	92.99 ± 5.27	83.93 ± 10.55

Data are presented as the average ± SD. pD$_2$: –Log of the average of the molar concentration that produced 50% of the maximum relaxation induced by PHAR-DBH-Me (10^{-12}–10^{-5} M). Max Tension corresponds to the average percentage of the tension decrease compared to the maximum tension developed in response to phenylephrine (PHEN) (10^{-6} M). PHAR-DBH-Me: control group; PHAR-DBH-Me + Indo: PHAR-DBH-Me in presence of indomethacin (10^{-6} M); PHAR-DBH-Me + L-NAME: PHAR-DBH-Me in presence of L-NAME (300 μM); PHAR-DBH-Me + Indo + L-NAME: PHAR-DBH-Me in presence of indomethacin (10^{-6} M) plus L-NAME (300 μM). *Denotes that the differences are significant respect to the control group ($p < 0.05$). n = 5 rats in each group.

and with the anesthetized animal than when the measurement is made indirectly. The difference in these results could be explained because this experimental maneuver generates stress in the animals, despite being trained for one week to measure BP. We observed that the recording of BP in basal control conditions was consistently higher with the indirect method.

As a cannabinoid receptor agonist [16], the hypotensive effect of the PHAR-DBH-Me could be due, in part, to the release of nitric oxide. Activation of the CB$_1$ and CB$_2$ receptors has been described to produce, among other effects, the release of nitric oxide and the activation of cyclic guanosine monophosphate/Protein kinase G [17,18]. Recently the CB$_1$ receptor was identified in mitochondria being able to activate G$_{\alpha i}$ proteins, causing the inhibition of adenylyl cyclase, with the consequent inhibition of protein kinase

A and the phosphorylation-dependent on specific subunits of the mitochondrial electron transport system, leading to a decrease in cellular respiration and heart function [19,20].

It has been reported that CB$_1$ and CB$_2$ receptors are coupled to G proteins mainly of the inhibitor G$_{i/o}$ type, through whose subunits α inhibit adenylyl cyclase, which results in a decrease in intracellular adenosine monophosphate levels [21,22]. Typical intracellular events mediated by G$_{i/o}$ proteins linked to CB$_1$ receptor activation could be another component of the hypotensive effect of PHAR-DBH-Me causing inhibition of voltage-dependent calcium channels, including P/Q, N, and L types present in vascular smooth muscle cells, as well as increased conductance to potassium [23,24].

Some studies have evaluated the effect of anandamide on blood

pressure using hypertensive rats. In these studies, a decrease in blood pressure was found when anandamide was administered or when its degradation was blocked by the administration of FAAH inhibitors. Furthermore, it was found in hypertensive but not normotensive rats, the administration of CB_1 receptor antagonists caused an increase in blood pressure and these animals showed increased expression of CB_1 receptors on cardiac and aortic endothelial cells [25,26].

In a previous study, it was found in normotensive animals, the decrease in blood pressure induced by anandamide (CB_1 receptor agonist) was mainly due to a decrease in cardiac output [27]. However, also found that in anesthetized hypertensive rats, the hypotensive effect was due in part to decreased peripheral vascular resistance. Our work provides evidence that PHAR-DBH-Me causes a significant decrease in blood pressure in both contexts. However, future research is required to study further the systemic effects of PHAR-DBH-Me to determine if the hypotensive effect is due to a synergistic effect of cardiac inotropism and decreased peripheral vascular resistance.

Endocannabinoids have been reported cardioprotective effects against cardiac ischemia secondary to hypoperfusion. In isolated rat hearts, 2-arachidonoylglycerol has a protective effect against cardiac ischemia, limiting infarct size in hearts undergoing periods of ischemia, and these same effects are observed when synthetic CB_1 and CB_2 receptor agonists were used [27]. Another study found that anandamide is able to limit infarct size in isolated hearts subjected to periods of ischemia [28]. In this same study, they reported that anandamide decreases the duration and amplitude of action potentials, and a CB_1 receptor antagonist abolishes these effects in myocardial cells of the rat. Furthermore, anandamide decreases the influx of calcium through L-type calcium channels, and this effect also appears to be CB_1 receptor-dependent [29].

PHAR-DBH-Me causes a significant increase in coronary flow in isolated rat hearts using the Langendorff preparation, and that this effect is dose-dependent. Like other endocannabinoids, PHAR-DBH-Me directly affects coronary blood flow, which could be related to endothelial nitric oxide synthesis/release. However, future studies are needed to evaluate the effects of PHAR-DBH-Me on the heart, primarily on cardiac automatism, cardiac inotropism, and other electrophysiological parameters of the myocardial cells, since as previously mentioned, the hypotensive effect induced by PHAR-DBH-Me may be due, at least in part, to decreased cardiac output.

The compound PHAR-DBH-Me induced in the aortic rings a relaxant response, which was dependent on the presence of endothelium and concentration. This vasodilator effect agrees with other results observed in mesenteric arteries of rats exposed to oleamide [30], where the location of the CB_1 receptor has been demonstrated, in addition to human pulmonary arteries [31,32].

The relaxing effect of PHAR-DBH-Me could be mediated mainly by the synthesis/release of endothelial nitric oxide because this effect was inhibited by the addition of L-NAME to the superfusion solution, as showed in the results. Nitric oxide has been reported to be involved in the endocannabinoid-induced vasodilatory response in different mammalian vessels [33,34]. On the other hand, it has been proposed that cannabinoid receptor agonists induce phosphorylation of endothelial nitric oxide synthase and increase nitric oxide synthesis. The addition of the inhibitor of nitric oxide synthesis, L-NAME, significantly decreases the relaxant response induced by these compounds [35].

The addition of a CB_2 cannabinoid receptor antagonist, but no CB_1 cannabinoid receptor antagonist, significantly decreased the PHAR-DBH-Me response, this suggest that the relaxant effect caused by this compound is mediated by CB_2 rather CB_1 receptor. CB_2 receptors are expressed in peripheral tissues in higher concentration than CB_1 receptors, as previously reported [36], and may induce nitric oxide release after activation in the endothelium [37], this relates to the endothelium dependence of this compound. This data contrasts with previously reported for oleamide, in resistance vessels had effects through the CB_1 receptor, whereas the CB_2 receptor was not involved [15], however, PHAR-DBH-ME had its effects on conductance vessels activating the CB_2 receptor, this difference could be explained by the type of blood vessel used.

Indomethacin was used to analyze the participation of vasoactive prostanoids synthesized through the cyclooxygenase pathway on the relaxing response induced by PHAR-DBH-Me. The relaxant response PHAR-DBH-Me-induced in the aortic rings with endothelium and indomethacin was significantly greater than preparations had administered PHAR-DBH-Me only. In aortic rings without endothelium, no effect was observed. These findings suggest the possibility the PHAR-DBH-Me induces the release of some endothelial prostanoid with vasoconstrictive effects, whose synthesis/release is blocked by the addition of indomethacin; this is related to what has been reported [38].

Finally, the fact that the addition of L-NAME plus indomethacin does not significantly modify the response to PHAR-DBH-Me in aortic rings without endothelium but in rings with endothelium increases the relaxant response suggests that this compound could stimulate an additional mechanism involved in the regulation of vascular tone NO-independent by the endothelium, such as endothelium-derived hyperpolarizing factor [39,40]. This is related to a previously reported for endocannabinoid-like molecule N-oleoylethanolamine, in which inhibition of cyclooxygenase and nitric oxide synthase potentiates their relaxing effect [41].

In the present study, we provide evidence that PHAR-DBH-Me has a hypotensive effect, increase flow coronary, and induces an endothelium- and concentration-dependent relaxant response in rat aortic rings through the cannabinoid CB_2 receptor. These effects appear to be related to nitric oxide synthesis and the production of contractile prostanoid. Further research is needed to understand the interaction between all the mechanisms involved

in the cardiovascular responses induced by PHAR-DBH-Me.

FUNDING

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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