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ORIGINAL ARTICLE **OPEN ACCESS**

Biological activity Exerted by an (-)-Epicactechin Derivative in an Ischemia-Reperfusion injury Model

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ABSTRACT
Some flavonoids have been used as cardioprotective drugs on myocardial ischemia-reperfusion injury; however their activity and the molecular mechanism involved on myocardial ischemia-reperfusion injury are very confusing. Therefore, the aim of this study was to evaluate the activity of (-)-epicatechin and their derivative on myocardial ischemia/reperfusion injury using an isolated heart model. Additionally, molecular mechanism involved in the activity exerted by the (-)-epicatechin derivative on perfusion pressure and coronary resistance was evaluated by measuring left ventricular pressure in absence or presence of following compounds; prazosin, propanolol, metoprolol, indomethacin and nifedipine. The results showed that the (-)-epicatechin derivative reduce infarct size compared with the control conditions and (-)-epicatechin. In addition, other data showed that the (-)-epicatechin derivative significantly increases the perfusion pressure and coronary resistance in an isolated rat heart in comparison the control and (-)-epicatechin. Finally, other data indicate that the (-)-epicatechin derivative increase left ventricular pressure in a dose-dependent manner; however, this phenomenon was significantly inhibited by propanolol and metoprolol. In conclusion, all these data suggest that the (-)-epicatechin derivative exert a cardioprotective effect via β_1-adrenergic receptor and consequently induce changes in the cAMP. This phenomenon results in decrease of myocardial necrosis after ischemia and reperfusion.
Keywords: (-)-epicatechin, coronary resistance

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INTRODUCTION

There are several reports of the biological activity exerted by the flavonoids in the heart; for example, a study was conducted in a population of Kuna Indians which showed that regular consumption of cocoa (which has a high content of flavonoids) provides cardiovascular benefits [1]. In addition, another cohort study in 1169 patients indicates that flavonoids may be responsible for the reduced mortality after myocardial infarction [2]. Other data indicate that specifically a polyphenol such as the (-)-epicatechin decreased the myocardial injury by ischemia-reperfusion in a rat model [3]. In addition, other studies indicate that (-)-epicatechin can prevents hypertension, proteinuria, and vascular dysfunction [4, 5]. These experimental results indicate that (-)-epicatechin may confer cardioprotection in myocardial ischemic injury through of different molecular mechanisms. For example, a study showed that (-)-epicatechin decreased the myocardial ischemic injury via protein kinases (AKT and ERK) activation [6]. Other data indicate that (-)-epicatechin exerts protective effects against oxidative stress induced by isoproterenol which results in a reduction in cardiac tissue damage by eliminating free radicals and antioxidant effects [7]. Also, a report shows that *in vitro* treatment with (-)-epicatechin brings results in a significant increase in the activity of Ca^{2+}-ATPase in hypertensive patients as well as in normal subjects [8]. Other data indicate that (-)-epicatechin reduces blood pressure in humans via increase nitric oxide [9, 10]. However other data suggest that (-)-epicatechin induces calcium and translocation independent eNOS activation in arterial endothelial cells [11]. All these experimental results show different molecular

mechanisms involved in the activity of (-)-epicatechin on the heart; this phenomenon could be conditioned by the different experimental designs used or by the functional groups involved in the chemical structure of (-)-epicatechin; in this sense, there are data which suggest that hydroxyl groups play an important role in various biological activities exerted by the (-)-epicatechin in other biological systems [12-15]. Analyzing this hypothesis, the objective of this study was to evaluate the biological activity of a (-)-epicatechin derivative in an ischemia/reperfusion injury model.

METHODOLOGY

General methods for chemical synthesis of epicatechin derivative

The compounds evaluated in this study were purchased from Sigma-Aldrich Co. Ltd. The melting points for the different compounds were determined on an Electrothermal (900 model). Infrared spectra (IR) were recorded using KBr pellets on a Perkin Elmer Lambda 40 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in chloroform ($CDCl_3$) using TMS as internal standard. EIMS spectra were obtained with a Finnigan Trace GCPolaris Q. spectrometer. Elemental analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of (((2R,3S)-2-(3,4-bis((tert-butyldimethylsilyl)oxy)phenyl)chromane-3,5,7-triyl)tris(oxy)) tris(tert-butyldimethysilane).

A solution of (-)-epicatechin (100 mg, 0.34 mmol), *tert*-Butyldimethylsilyl chloride (200 µl, 1.07 mmol) in 5 ml chloroform was stirred for 72 h at room temperature. After, the organic phase was evaporated to dryness under reduced pressure. Then, the residue was purified by crystallization from methanol:water (1:2) yielding 87 % of product, m.p. 178-180 ^{o}C; IR (Vmax, cm^{-1}): 1096; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 0.08 (s, 6H), 0.20 (s, 6H), 0.22 (s, 6H), 0.26 (s, 6H), 0.70 (s, 9), 0.88 (s, 9H), 1.00 (s, 18H),, 2.80-4.90 (m, 4H), 5.96-6.14 (m, 2H), 6.80-7.12 (m, 3H) ppm. ^{13}C NMR (75.4 Hz, $CDCl_3$) δ_C: -4.98, -4.70, -4.26, 2.86, 18.00, 18.20, 18.62, 18.80, 24.72, 25.52, 25.60, 25.76, 30.00, 71.02, 82.80, 93.92, 99.28, 101.32, 114.80, 118.50, 119.06, 128.82, 144.66, 146.51, 153.22, 154.00, 156.28 ppm. EI-MS *m/z*: 860.51. Anal. Calcd. for $C_{45}H_{84}O_6Si_5$: C, 62.73; H, 9.83; O, 11.14; Si, 16.30. Found: C, 62.64; H, 9.70.

Biological evaluation

Methods

The experimental methods used in this investigation were reviewed and approved by the Animal care and use Committee of University Autonomous of Campeche (No. PI-420/12) and were in accordance with the guide for the care and use of laboratory animals [16].

Animals

Male Wistar rats; weighing 200-250 g were obtained from University Autonomous of Campeche

Reagents

The compounds involved in this study were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v).

Experimental design

Male rats (200-250 g) were anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/Kg body weight. After, the chest was opened, and a loose ligature passed through the ascending aorta. Then, the heart was removed and immersed in ice cold physiologic saline solution*. The hearts were trimmed of non-cardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. Following, an initial perfusion rate of 15 ml/min for 5 min was followed by a 15 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

*Physiologic saline solution (Krebs-Henseleit solution) was composed of; NaCl, 117.8 mmol; KCl, 6 mmol; $CaCl_2$, 1.75 mmol ; NaH_2PO_4, 1.2 mmol ; $MgSO_4$, 1.2 mmol ; $NaHCO_3$, 24.2 mmol ; glucose, 5 mmol; and sodium pyruvate 5 mmol. and the coronary flow was adjusted with a variable speed peristaltic pump.

*Krebs-Henseleit solution was actively bubbled with a mixture of O_2/CO_2 (95:5/5 %) and regulated at a pH of 7.4 and 37°C.

Perfusion pressure

The biological activity exerted by the drugs involved in this study on perfusion pressure was assessed using a pressure transducer connected to the chamber where the hearts were placed. In addition, the transducer was also linked to a system of computerized data capture (Biopac).

Evaluation of left ventricular pressure

The contractile activity was evaluated by measuring left ventricular developed pressure (LV/dP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium [17]. It is important to mention that the latex balloon was linked to a cannula which was bound to a pressure transducer that was connected with the MP100 data acquisition system.

First stage

Evaluation of activity of the (-)-epicatechin derivative on the ischemia/reperfusion injury

The hearts were subjected to ischemia for 30 minutes by turning off the perfusion system. Then of this period the system was restarted and the hearts were reperfused by 30 minutes with Krebs-Henseleit solution. The hearts were randomly divided into 2 treatment groups with n = 9 as following:

Group I.

Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs- Henseleit solution).

Group II.

The (-)-epicatechin was administered before ischemia period (for 10 minutes) and during the entire period of reperfusion.

Group III.

The (-)-epicatechin derivative was administered before ischemia period (for 10 minutes) and during the entire period of reperfusion. At the end of each experiment, the perfusion pump was stopped and 0.5 ml of fluorescein* solution (0.10%) was injected slowly through a sidearm port connected to the aortic cannula. The dye was passed through the heart for 10 sec to ensure its uniform tissue distribution. After, the heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. It is important to mention that the areas of the normal left ventricle non risk region, area at risk, and infarct region were determined using methods previously reported [18]. Total area at risk was expressed as the percentage of the left ventricle.

*The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region.

Second stage

Effect induced by the (-)-epicatechin derivative on perfusion pressure: The activity of (-)-epicatechin derivative on in perfusion pressure was evaluated at a concentration of 0.001 nM in periods different (3 to 18 min). It is noteworthy that the effects were obtained in isolated hearts perfused at a constant-flow rate of 10 ml/min.

Evaluation of the activity exerted by the (-)-epicatechin derivative on coronary resistance: The effect of the (-)-epicatechin derivative at a concentration of 0.001 nM on coronary resistance was evaluated. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min. It is important to mention that changes in coronary pressure reflected the changes in coronary resistance.

Third stage

Effects of the (-)-epicatechin derivative on left ventricular pressure through the calcium channel: Intracoronary boluses (50 µl) of (-)-epicatechin derivative [0.001 to 100 nM] were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of nifedipine at a concentration of 1 nM (duration of the pre-incubation with nifedipine was for a period of 10 min).

Effect exerted by the (-)-epicatechin derivative on left ventricular pressure via prostanglandins. The (-)-epicatechin derivative [0.001 to 100 nM] was administered and the corresponding activity on the left ventricular pressure was evaluated. The experimental (curve-control) was repeated in the presence of indomethacin at a concentration of 1 nM (duration of the pre-incubation with indomethacin was for a period of 10 min).

Effects induced by the (-)-epicatechin derivative on left ventricular pressure through α_1-adrenergic receptor. The (-)-epicatechin derivative (0.001 to 100 nM) was administered and the corresponding effect on the left ventricular pressure was determined. This process (curve-control) was repeated in the presence of prazosin at a concentration of 1 nM (duration of preincubation with prazosin was by a 10 min equilibration period).

Effects induced by the (-)-epicatechin derivative on left ventricular pressure through β_1-adrenergic receptor. The (-)-epicatechin derivative (0.001 to 100 nM) was administered and the corresponding effect on the left ventricular pressure was determined. The experimental (curve-control) was repeated in the presence of propanolol or metoprolol at a concentration of 1 nM (duration of preincubation with propanolol or metoprolol was by a 10 min equilibration period).

Effect of the (-)-epicatechin derivative on cAMP levels. The hearts were perfused* with the (-)-epicatechin derivative (1 nM) and vehicle (control) for 2, 5, or 30 min.

Effect of the isoproterenol on cAMP levels. The hearts were perfused* with ***isoproterenol*** (1 nM) and vehicle (control) for 2, 5, or 30 min.

*After the appropriate period of infusion, atrial tissue was removed, and the ventricles were immediately frozen with liquid nitrogen and stored at 270 °C until assayed. Tissue samples were homogenized with

6% trichloroacetic acid at 4 °C to give a 10% (w/v) homogenate, followed by centrifugation at 2000 rpm (Hettich Zentrifugen, EBA-270) for 15 min. Then supernatants were collected and washed with water:diethyl ether (5:1 v/v) five times. The extracts were lyophilized and processed for the measurement of cAMP content by use of a standard 125 I radioimmunoassay kit supplied by Amersham International [19].

Statistical analysis

The obtained values are expressed as average ± SE, using each heart (n = 9) as its own control. The data obtained were put under Analysis of Variance (ANOVA) with the Bonferroni correction factor using the SPSS 12.0 program [20]. The differences were considered significant when ***p*** was equal or smaller than 0.05.

RESUSLTS AND DISCUSSION

Evaluation Chemistry

The first stage was accomplished by protecting of the hydroxyl groups from (-)-epicatechin (Fig. 1). It is important to mention that several triorganosilyl groups have been employed for protection of hydroxyl groups such as tert-butyldimethylsilyl and tert-butyldiphenylsilyl [21]; in this study, (-)-epicatechin reacted with tert-butyldimethylsilyl chloride to form the compound **2**; it is noteworthy that with this method there are very yielding. The structure of **2** was confirmed using IR and NMR spectroscopy. The IR spectra contained characteristic vibration at 1096 for eter groups for the (-)-epicatechin derivative. The ^{1}H NMR spectrum of the compound **2** shows signals at 0.20-0.22, 0.70 and1.00 ppm for methyl groups involved in the *tert*-butyldimethylsilane fragment bound to A-ring; 0.08 and 0.88 ppm for methyl groups of *tert*-butyldimethylsilane fragment bound to B-ring; at 0.26 an 1.00 ppm for methyl groups of *tert*-butyldimethylsilane fragment bound to C-ring; at 2.80-4.90 ppm for methylene groups of B-ring; at 5.96-6.14 ppm for methylene groups of A-ring; at 6.80-7.12 ppm for methylene groups of C-ring. The ^{13}C NMR spectra displays chemical shifts at -4.70, -4.26, 18.00, 18.62 and 25.52-25.60 ppm for methyl groups of *tert*-butyldimethylsilane fragment of A-ring; at -4.98 and 18.80-24.72 ppm for methyl groups of *tert*-butyldimethylsilane fragment of B-ring; at 2.86, 18.20 and 25.76 ppm for methyl groups of *tert*-butyldimethylsilane fragment of C-ring; 93.92-101.32 and 153.22-156.28 ppm for carbons of A-ring; at 30.00-82.80 ppm for methylene of B-ring; at 114.80-146.51 ppm for carbons of C-ring. Finally, the presence of the (-)-epicatechin derivative was confirmed with mass spectrum which showed a molecular ion at *m/z* 860.51.

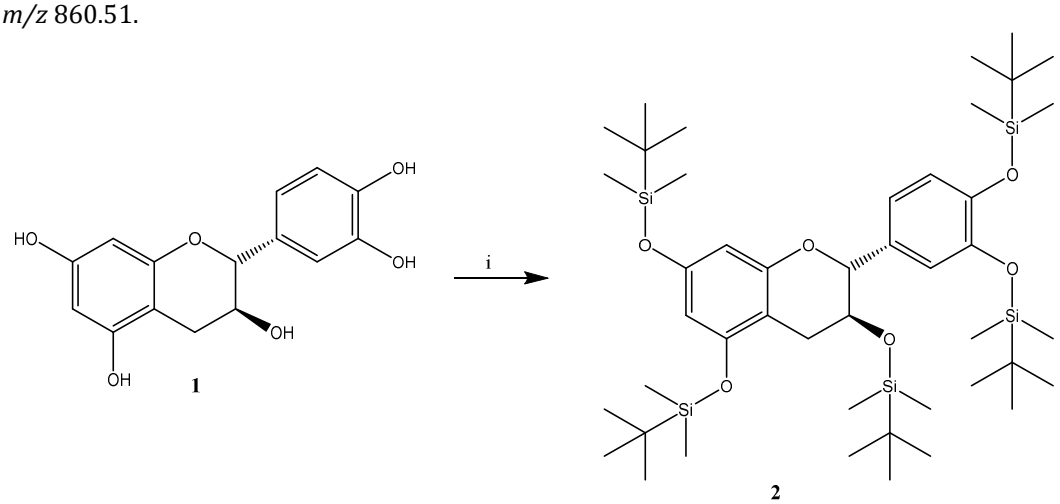

Fig. 1. Synthesis of the (-)-epicatechin derivative (**2**). Reaction of (-)-epicatechin with tert-butyldimethylsilyl chloride (i) to form the compound 2.

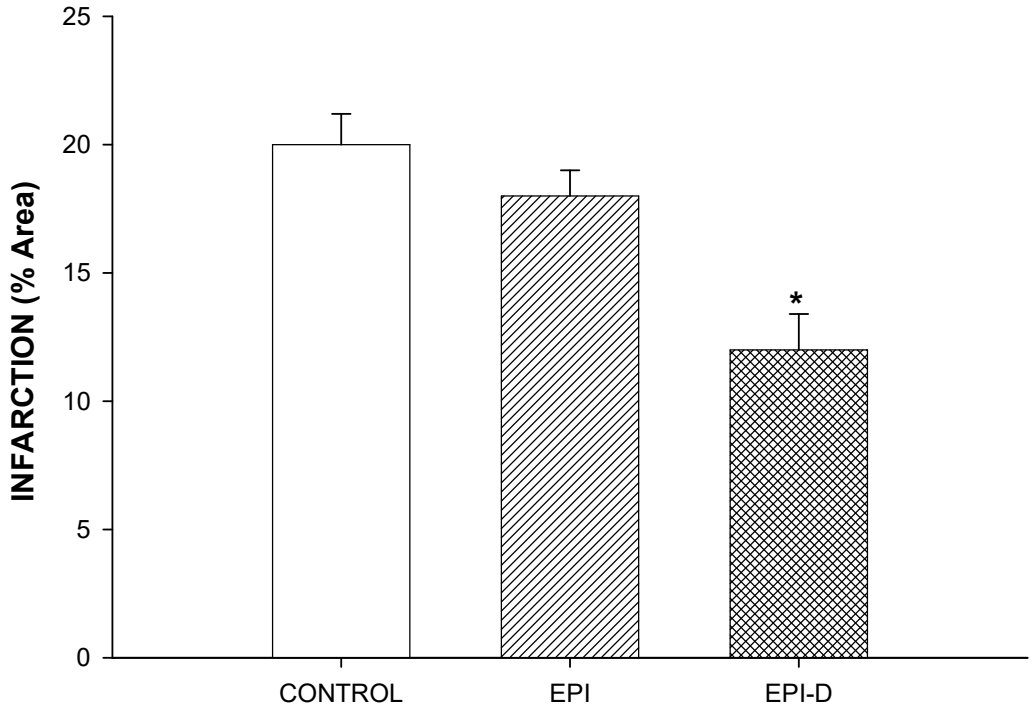

Figure 2. Effect exerted by EPI-D at a dose of 1 nM on the functional recovery of rat hearts subjected to ischemia and reperfusion in comparison with EPI [0.01 nM] and control conditions. EPI = (-)-epicatechin; EPI-D = (-)-epicatechin derivative.

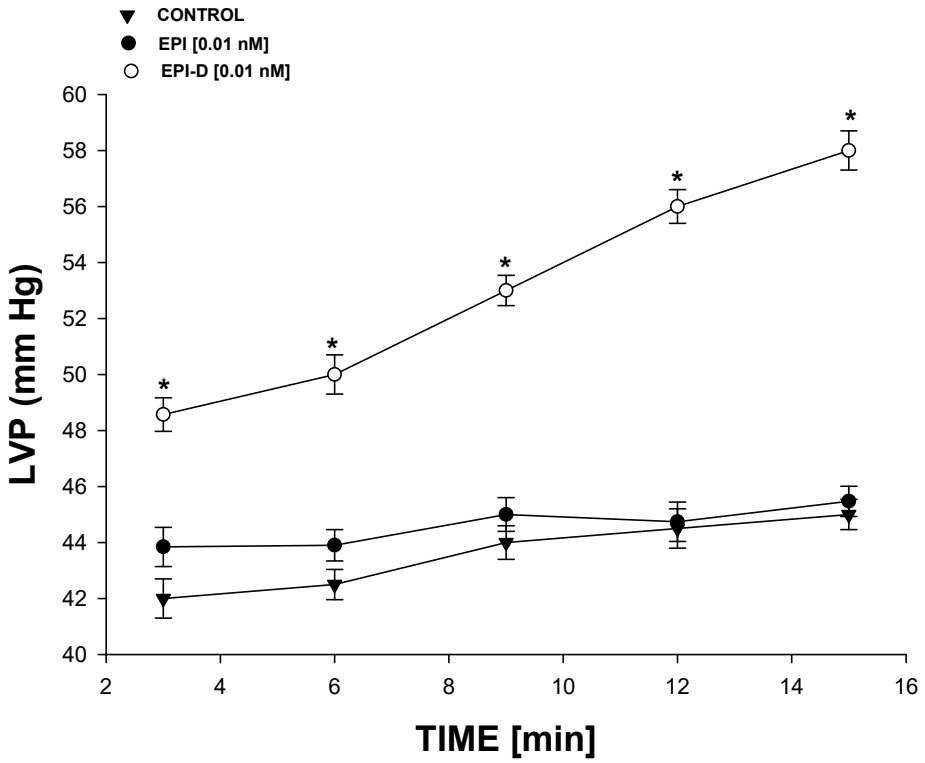

Figure 3. ***Effect induced by*** the EPI-D ***on perfusion pressure.*** The results show that EPI-D significantly increase perfusion pressure (***p*** = 0.06) through time in comparison with (-)-epicatechin (EPI) and the control conditions. Each bar represents the mean ± S.E. of 9 experiments. EPI-D = (-)-epicatechin derivative.

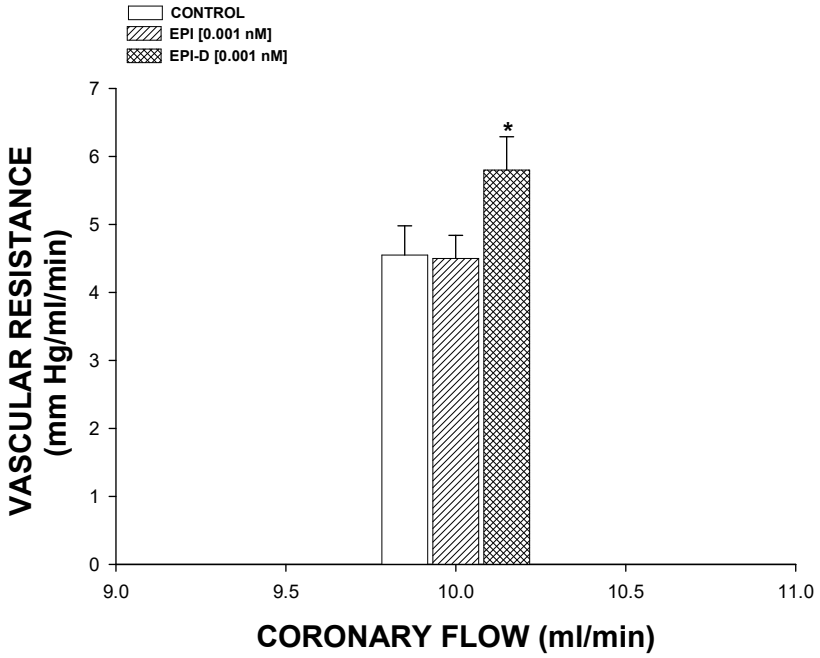

Figure 4. ***Activity exerted by*** the EPI-D ***on coronary resistance.*** The results show that coronary resistance was higher (***p*** = 0.05) in the presence of the EPI-D derivative in comparison with the control conditions and (-)-epicatechin (EPI). Each bar represents the mean ± S.E. of 9 experiments. EPI-D = (-)-epicatechin derivative

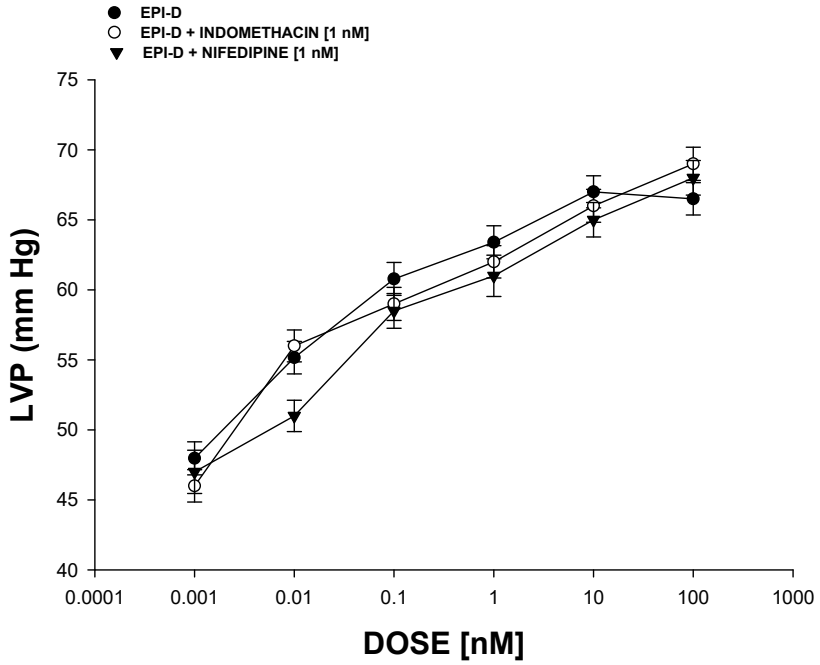

Figure 5. ***Effect exerted by EPI-D on left Ventricular Pressure (LVP) through prostaglandins synthesis or calcium channel activation*.** EPI-D [0.001 to 1 nM] was administered (intracoronary boluses, 50 µl) and the corresponding effect on the LVP was evaluated in absence and presence of indomethacin or nifedipine at a dose of 1 nM. The results showed that activity induced by EPI-D on LVP was not inhibited in presence of indomethacin or nifedipine. Each bar represents the mean ± S.E. of 9 experiments. EPI-D = (-)-epicatechin derivative.

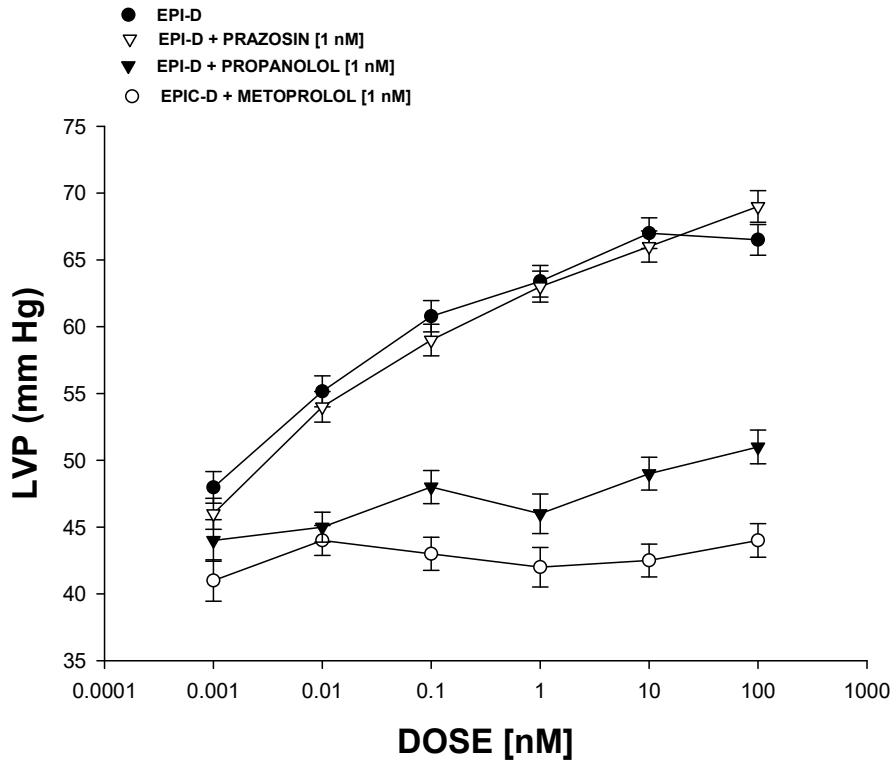

Figure 6. ***Effects induced by*** EPI-D ***on LVP via adrenergic system.*** Intracoronary boluses (50 µl) of EPI-D [0.001 to 100 nM] were administered and the corresponding effect on the LVP was determined in the absence and presence of prazosin or propanolol or metoprolo. The results showed that EPI-D increase the LVP in a dependent dose manner and this effect was not inhibited in the presence of prazosin. However, the activity induced by EPI-D on LVP was blocked by and propanolol or metoprolol. Each bar represents the mean ± S.E. of 9 experiments. LVP = left ventricular pressure; EPI-D = (-)-epicatechin derivative.

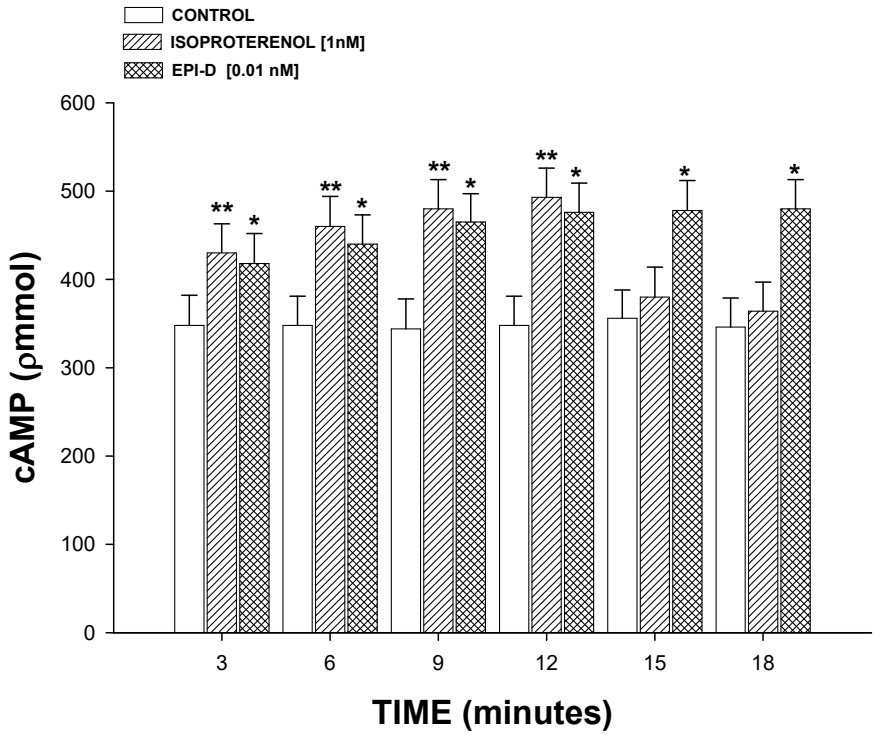

Figure 7. ***Effect exerted by EPI-D and isoproterenol on cAMP levels through of time.*** The results show that cAMP levels were higher (p = 0.06) in the presence of isoproterenol (3-12 min) in comparison with EPI-D and the control conditions. However, EPI-D increased the concentration for cAMP after of the 15 min compared with isoproterenol and control. Each bar represents the mean ± S.E. of 6 experiments

Biological evaluation

Several studies indicate that activity of (-)-epicatechin on different biological system may depend of hydroxyl groups involved in their chemical structure [12-15]. Analyzing this hypothesis in this study, the biological activity of (-)-epicatechin and its derivative was evaluated in an ischemia-reperfusion injury model. The results shown in the figure 2 indicate that biological activity exerted by the (-)-epicatechin derivative (EPI-D) can reduced infarct size (expressed as a percentage of the area at risk) in comparison with vehicle-treated hearts and (-)-epicatechin. This phenomenon could be because EPI-D exerts some influence on blood pressure which could result reduction in the infarct size, and decrease the myocardial injury after ischemia-reperfusion.

Second stage

Analyzing the hypothesis above mentioned, in this study the biological activity exerted by the (-)-epicatechin and their derivative on perfusion pressure and coronary resistance in isolated rat heart model was evaluated. The results showed that the EPI-D (0.001 nM) increased significantly ($p = 0.06$) the perfusion pressure (Figure 3) as a consequence of changes in the time (3-18 min) in comparison with the control conditions and (-)-epicatechin. Other data shown in the Figure 4 indicate that EPI-D increased the coronary resistance* (***p*** = 0.05) compared with (-)-epicatechin and the control conditions. All these data indicate; 1) the hydroxyl groups are not essentials for biological activity of EPI-D on ischemia-reperfusion injury; 2) the biological activity of EPI-D on perfusion pressure and coronary resistance could be the result of interaction of EPI-D with some vasoactive substances which exert some change in blood pressure or coronary resistance such as happening with other type of compounds [22, 23].

*Coronary resistance was calculated as the ratio of perfusion pressure at coronary flow assayed (10ml/min).

Third stage

Analyzing these data and other reports which indicate that some drugs exert its effect on left ventricular pressure (LVP) through prostaglandin synthesis [24]; in this study the activity exerted by EPI-D on left ventricular pressure in the absence or presence of indomethacin was analyzed to evaluate the possibility that the activities exerted by the EPI-D involve stimulation and secretion of prostaglandins.. The results showed (Figure 5) showed that EPI-D increase LVP in a dose dependent manner [0.001 nM] and this effect was not inhibited in presence of indomethacinn (***p*** = 0.05) at a concentration of 1 nM. These results indicate that activity exerted by the EPI-D derivative on left ventricular pressure was not via prostanoids synthesis and secretion.

In the search of other possible molecular mechanism involved in the effect exerted by EPI-D on the left ventricular pressure; also were analyzed some reports, which indicate that some compounds exert changes on left ventricular pressure via activation the calcium channel [25]. In order to evaluate this premise, in this study the effect exerted by EPI-D on calcium channel *type*-L was evaluated in absence or presence of nifedipine. The results showed that, the effect exerted by EPI-D was not blocked by nifedipine, this phenomenon indicated that biological activity of EPI-D is no via calcium channel activation.

On the other hand, in the search of possibly molecular mechanism involved in the effect induced by EPI-D and analyzing some data which suggest that other type of compounds exert their effect through of adrenergic receptors [26, 27], in this study the effect exerted by the EPI-D on left ventricular pressure was evaluated using prazosin as pharmacological tool to characterize whether this mechanism was involved in their pharmacological activity. The results showed that EPI-D increases the left ventricular pressure in a dose dependent manner; however this effect was not inhibited in presence of prazosin. These data indicate that the activity exerted by EPI-D was through activation of α_1-receptor.

In the following stage, the biological activity of EPI-D on LVP was evaluated in presence or absence of propanolol and metoprolol. The results showed that effect exerted by EPI-D on LVP was blocked by propanolol and metoprolol. These data indicate that activity induced by EPI-D on LVP was via β_1-receptor adrenergic. However, in the search of synthesis or release of some vasoactive substance by β_1-receptor adrenergic activation, in this study other reports were analyzed which suggest that some compounds exert their biological activity on LVP involves changes in the levels of $_c$AMP [28]. To evaluate this hypothesis, other experiments were made to assess whether the positive inotropic activity induced by EPI-D on left LVP could induce changes on $_c$AMP levels using as pharmacological tool to isoproterenol. The results indicate that EPI-D increases $_c$AMP levels in a manner time-dependent compared with isoproterenol and control conditions; however, it is important to mention that after 15 to 18 minutes the effect of isoproterenol decreased significantly; these phenomenon is similar a to other studies previously reported for isoproterenol [29, 30]. All these data indicate; 1) the effect exerted by EPI-D is mediated by cAMP in a manner time-dependent; 2) The effect of EPI-D is higher in comparison with isoproterenol.

Here it is important to mention that this phenomenon could be the result of degree of lipophilicity of EPI-D may be higher in comparison with isoproterenol.

CONCLUSION

Changes in the functional groups of (-)-epicatechin induce different biological activity in an ischemia/reperfusion injury. This phenomenon indicate that hydroxyl groups in this biological model are not essentials for biological activity of the (-)-epicatechin-derivative. On the other hand, the (-)-epicatechin derivative is a particularly interesting drug, because the activity induced for this compound on injury by ischemia/reperfusion involves a molecular mechanism different in comparison with other drugs. This phenomenon may constitute a novel therapy for ischemia/reperfusion injury.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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