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Full Length Research Paper

Design and synthesis of an estradiol derivative and evaluation of its inotropic activity in isolated rat heart

Figueroa-Valverde L.[1]*, Díaz-Cedillo F.[2], López-Ramos M.[1], García-Cervera E[1] and Pool-Gómez E.[1]

[1]Laboratory of Pharmaco-Chemistry, Faculty of Chemical Biological Sciences, Universidad Autonóma de Campeche, Av. Agustín Melgar s/n, Col Buenavista C.P.24039 Campeche Cam., México.
[2]Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional. Prol. Carpio y Plan de Ayala s/n Col. Santo Tomas, México, D.F. C.P. 11340.

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Several studies indicate that some steroid derivatives have inotropic activity; nevertheless, there is scarce information about the effects of the estradiol derivatives at cardiovascular level. Therefore, in this study, estradiol derivative was synthetized with the objective of evaluating its inotropic activity. In this first stage, the Langendorff technique was used to measure perfusion pressure and coronary resistance changes in isolated rat heart in absence or presence of estradiol derivative. In second stage, the inotropic activity of estradiol derivative was evaluated by measuring left ventricular pressure in absence or presence of following compounds; tamoxifen, prazosin, metoprolol, indomethacin and nifedipine. The results showed that the estradiol derivative significantly increase the perfusion pressure and coronary resistance in isolated heart. Additionally, other data indicate that estradiol derivative increase left ventricular pressure in a dose-dependent manner [10^{-9} to 10^{-4} mmol]; nevertheless, this phenomenon was significantly inhibited by nifedipine at a dose of 1×10^{-6} mmol. In conclusion, these data suggest that the estradiol derivative induces positive inotropic activity through of activation the L-type calcium channel.

Key words: Estradiol derivative, Langendorff, inotropic activity.

INTRODUCTION

There are reports which indicate that congestive heart failure (CHF) is a main cause of death in patients with heart disease (Katz et al., 1986; Schunkert et al., 1999; Braunwald and Bristow, 2000). Several drugs have been used for the treatment of CHF such as the digitalis glycosides. Unfortunately, the use of these agents is limited by their narrow therapeutic window and their propensity to cause life-threatening arrhythmias (Kersten et al., 2000; Silverberg et al., 2000). In this sense, there has been a resurgence of interest in cardiotonic steroids derivatives, it is important to mention that these molecules exert a large number of effects in cardiac tissue (Lederer and Tsien, 1976; Wier and Hess, 1984). For example, the strophanthidin (steroid derivative) increase the force of contractionby changes in the calcium levels (Clark, 1914; Hart et al., 1983). In addition, there are studies that show the synthesis of a steroid derivative (F90927) which exerts a positive inotropic activity in cardiac muscle via activation of the L-type Ca^{2+} channel (Pignier et al., 2006). Additionally, a series of steroid derivatives (Gobbini et al., 2001; De munari et al., 2003) were synthesized which showed a positive inotropic effect, mainly by inhibition of Na^{+}, K^{+}-ATPase. Nevertheless, other reports indicate that 14β-

*Corresponding author. E-mail: lauro_1999@yahoo.com. Tel: 9818119800 Ext. 73006.

Abbreviations: CHF, Congestive heart failure; **IR,** infrared spectra; **TMS,** tetramethylsilane; **NMR,** nuclear magnetic resonance; **EIMS,** electron impact mass spectrometric; **UAC,** Universidad Autonoma de Campeche; **LVdP,** left ventricular developed pressure; **LVP,** left ventricular pressure.

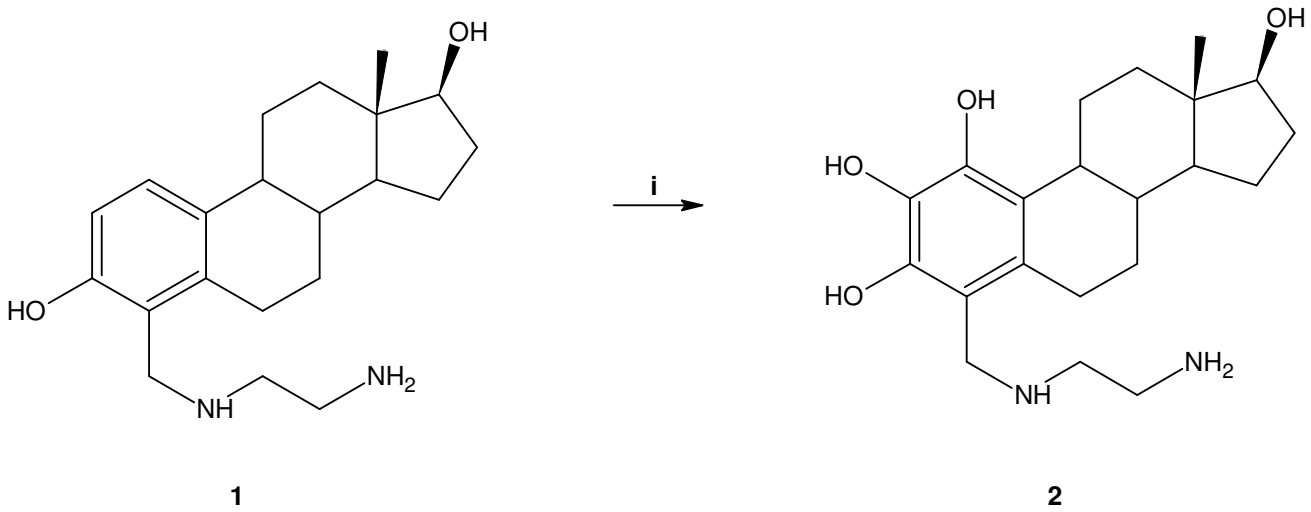

Figure 1. Synthesis of 4-[(2-Amino-ethylemino)-methyl]13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]-phenanthrene-1,2,3,17-tetraol (2); ii) Cl2/AlCl3, H+.

hydroxyprogesterone (Templeton et al., 1987) increases the contractility of isolated cardiac tissue via glycoside receptor. Additionally, it is important to mention that recently a furosemide-pregnenolone derivative was synthetized which induce positive inotropic activity in cardiac muscle via activation of the L-type Ca^{2+} channel (Figueroa et al., 2011a). All these data show that several steroid derivatives induce inotropic effects in the cardiovascular system; nevertheless, the cellular site and molecular mechanism involved in its inotropic activity are very confuse, perhaps this phenomenon is due to differences in the chemical structure of the steroid derivatives. Therefore, data information is needed to characterize the activity induced by steroid derivatives at cardiovascular level. To provide this information, the present study was designed to investigate the effects of an estradiol derivative on perfusion pressure and vascular resistance in isolated rat hearts using the Langendorff technique. In addition, to evaluate the molecular mechanism involved in the inotropic activity induced by the steroid-derivative on left ventricular pressure the following compounds were used as pharmacological tools; tamoxifen [antagonist of estrogen receptor] (Shiau et al., 1998), prazosin [α_1 adrenoreceptor antagonist] (Graham et al., 1977), metroprolol [selective β_1 receptor blocker] (Bengtsson et al., 1975) and nifedipin [antagonist of calcium-channel] (Henry, 1980).

MATERIALS AND METHODS

Chemical synthesis

Estradiol-ethylenediamine derivative (Compound 1; Figure 1) was prepared according to a previously reported method by Figueroa (2011b) and the other 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 nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in DMSO-d_6 using tetramethylsilane (TMS) as internal standard. Electron impact mass spectrometric (EIMS) spectra were obtained with a Finnigan Trace GCPolaris Q. spectrometer. Elementary analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of 4-[(2-Amino-ethylemino)-methyl]13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]-phenanthrene-1,2,3,17-tetraol (4)

A flask (I) with a solution of 1 (100 mg, 0.29 mmol), hydrochloric acid (0.5 ml) concentrate, anhydrous aluminium trichloride (80 mg, 0.60 mmol) in 20 ml of methanol was connected with a glass tube to other flask (II) which had a solution of hydrochloric acid concentrate. The reaction was stirring for 24 h to room temperature. The reaction mixture was evaporatedto a smaller volume. After, water was added (30 ml) to the mixture obtained. Then the precipitate was washed 10 times with water and dried at 100°C.

Biological method

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of Universidad Autonoma de Campeche (UAC) and were in accordance with the Guide for the Care and Use of Laboratory Animals (Bayne, 1996). Male rats (Wistar; weighing 200-250 g) were obtained from UAC.

Reagents

All drugs were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v).

Langendorff technique

Briefly, the male rat (200 - 250 g) was anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/Kg body weight. Then the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in ice cold physiologic saline solution. The heart was trimmed of non-cardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. It is important to mention that perfusion medium was the Krebs-Henseleit solution (pH 7.4, 37°C) composed of (mmol); 117.8 NaCl; 6 KCl; 1.75 $CaCl_2$; 1.2 NaH_2PO_4; 1.2 $MgSO_4$; 24.2 $NaHCO_3$; 5 glucose and 5 sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95:5). The coronary flow was adjusted with a variable-speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 25 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

Induction of congestive heart failure (CHF)

CHF was development mainly of method previous reported (Figueroa, 2011c), in this process the pentobarbital (100/kg mg) was administered through of cannula inserted in the aorta to induce CHF.

Perfusion pressure

Evaluation of measurements of perfusion pressure changes induced by drugs administration in this study were assessed using a pressure transducer connected to the chamber where the hearts were mounted and the results entered into a computerized data capture system (Biopac).

Inotropic activity

Contractile function was assessed by measuring left ventricular developed pressure (LVdP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium. It is important to mention that latex balloon was bound to cannula which was linked to pressure transducerthat was connected with the MP100 data acquisition system.

Biological evaluation

First stage

Effect induced by the estradiol derivative on perfusion pressure: Changes in perfusion pressure as a consequence of increases in time (3-18 min) in absence (control) or presence of the estradiol derivative at a concentration of 1×10^{-9} mmol were determined. The effects were obtained in isolated hearts perfused at a constant-flow rate of 10 ml/min.

Evaluation of effects exerted by the estradiol derivative on coronary resistance: The coronary resistance in absence (control) or presence of the estradiol derivative at a concentration of 1×10^{-9} mmol was evaluated. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min. Since a constant flow was used changes in coronary pressure reflects the changes in coronary resistance.

Second stage

Effects induced by the estradiol derivative on left ventricular pressurethrough estrogen receptors: Intracoronary boluses (50 µl) of the estradiol derivative (10^{-9} to 10^{-4}mmol) were administered and the corresponding effect on the left ventricular pressure was determined. The dose-response curve (control) was repeated in the presence of tamoxifen at a concentration of 10^{-6}mmol (duration of preincubation with tamoxifen was by a 10 min equilibration period).

Effect exerted by the estradiol derivative on left ventricular pressurein the presence of α_1 adrenergic blocker: The boluses (50 µl) of the estradiol derivative [10^{-9} to 10^{-4} mmol] were administered and the corresponding effect on the left ventricular pressure was evaluated. It is important to mention that the bolus injection administered was done in the point of cannulation. The dose-response curve (control) was repeated in the presence of prazosin at a concentration of 10^{-6} mmol (duration of preincubation with prazosin was by a 10 min equilibration period).

Effects induced by the estradiol derivative on left ventricular pressurein the presence of β_1 adrenergic blocker: The boluses (50 µl) of estradiol derivative [10^{-9} to 10^{-4} mmol] were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of metoprolol at concentration of 10^{-6} mmol (duration of preincubation with metoprolol was by a 10 min equilibration period).

Effects of the estradiol derivative on left ventricular pressurethrough the calcium channel: Intracoronary boluses (50 µl) of estradiol derivative [10^{-9} to 10^{-4} mmol] 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 10^{-6} mmol (duration of preincubation with nifedipine was by a 10 min equilibration period).

Effects induced by the estradiol derivative on the intracellular calcium levels: Changes in calcium levels as a consequence of increases in time (3-18 min) induces by estradiol derivative (1×10^{-9} mmol) were evaluated. The dose-response curve (control) was repeated in the presence of nifedipine at a concentration of 10^{-6} mmol (duration of preincubation with nifedipine was by a 10 min equilibration period).

It is important to mention, that intracellular calcium was evaluated using the technique reported by Boe and Khan (1929). In this method, 5 mL of samples obtained of perfused (metabolic solution) were used to evaluate the intracellular calcium. It is important to mention that the samples were taken every 3 min (six times). After, 4 ml trifluoroacetic acid (10%) was added each to sample. This solution was mixed for 5 min at room temperature and after 1 mL of NaOH (25%) was added. To mixture 1 ml was trisodic phosphate added and the mixture was vortexed (1 min) and centrifuged to 4000 rpm (5 min). The supernatant was separated from the aqueous solution. After 5 mL of a buffer solution (pH = 10) and 3 ml of eriochromeblack was added to the aqueous solution. The mixture was titled with ethylenediaminetetraaceticacid (EDTA) (f = 0.847).

Statistical analysis

The obtained values are expressed as average ± SE, using each heart as its own control. The data obtained were put under an analysis of variance (ANOVA) using the Bonferroni correction factor (Hocht et al., 1999). The differences were considered significant when *p* was equal or smaller than 0.05.

RESULTS

Chemical synthesis

The yielding of estradiol derivative (Figure 1) was of 75%

Table 1. ^{1}H NMR (300 MHz, DMSO-d_6) data for the estradiol derivative.

δ_H:ppm
0.76 (s, 3H), 0.88 (m, 1H), 0.96-1.25 (m, 3H), 128-1.47 (m, 2H), 1.64-1.91 (m, 6H), 2.35-2.55 (m, 2H), 2.65 (t, 2H, J = 6 Hz), 2.76 (m, 1H), 2.79 (t, 2H, J = 6 Hz), 3.67 (m, 1H), 3.74 (s, 2H), 6.10 (broad, 7H).

Table 2. ^{13}C NMR (300 MHz, DMSO-d_6) data for the estradiol derivative.

δ_H:ppm
11.32 (C-18), 23.30 (C-9), 26.16 (C-5), 27.72 (C-10), 28.02 (C-11), 30.42 (C-8), 37.33 (C-6), 38.43 (C-3), 41.38 (C-26) , 43.64 (C-1), 44.63 (C-4), 44.81 (C-23), 50.53 (C-2), 53.73 (C-25), 81.66 (C-7), 109.10 (C-17), 122.08 (C-13), 125.03 (C-15), 127.82 (C-12), 139.39 (C-16), 145.30 (C-14).

with melting point of 90-92°C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) at 3380, 3330 and 3310. In addition, the chemical shifts of the spectroscopic analyses of ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) for the estradiol derivative are showed down.

Finally, the results of mass spectroscopy (MS) (70 ev) shown; *m/z* 376.30 [M^+] 273.30, 191.20 and 137.10. Additionally, the elementary analysis datafor the estradiol derivative ($C_{21}H_{32}N_2O_4$) were calculated (C, 66.99; H, 8.57; N, 7.44; 0, 17.00) and found (C, 70.02; H, 8.54).

Biological activity

First stage

In this study, the activity induced by the estradiol derivativeon perfusion pressure and coronary resistance in the isolated rats heart were evaluated. The results obtained from changes in perfusion pressure as a consequence of increases in the time (3 to 18 min) in absence (control) or in presence of estradiol derivative (Figure 2), showed that estradiol derivative [10^{-9} mmol] significantly increase the perfusion pressure(p = 0.005) in comparison with the control conditions [10^{-9} mmol]. Additionally, another result showed that coronary resistance, calculated as the ratio of perfusion pressure at coronary flow assayed (10 mL/min) was higher in the presence of estradiol derivative than in control conditions (p = 0.005) at a concentration of 10^{-9} mmol (Figure 3).

Second stage

Figure 4 shows that, the estradiol-ethylenediamine derivative induces anincrease in the perfusion pressure in a dose dependent manner [10^{-9} to 10^{-4} mmol] and that this effect was not inhibited by tamoxifen [10^{-6} mmol].

On the other hand, other experiments showed that estradiol derivative increase the perfusion pressure in a dose dependent manner [10^{-9} to 10^{-4} mmol] and this effect was not inhibited in presence of prazosin (Figure 5) or metroprolol (Figure 6) drugs at a concentration of 10^{-6} mmol.

Alternative experimental indicate that effect induced bythe estradiol derivative on perfusionpressure (Figure 7) in presence of nifedipine at aconcentration of 10^{-6} mmol was reduced significantly (p = 0.005).

Finally, other results (Table 3) showed that effect induced by the estradiol derivative [10^{-9} mmol] exert increase in the intracellular calcium levels as a consequence of increases in the time (3 - 18 min); nevertheless, this effect is significantly reduced (p = 0.005) in presence of nifedipine [10^{-6} mmol].

DISCUSSION

Synthesis chemical

Many procedures for hydroxylation of steroid derivatives are available in the literature. The most widely practiced methods employ enzymatic catalysis (Jellinck and Brown, 1971), trimethyl borate/H_2O_2 (Iriarte et al., 1958), osmium tetroxide (Kiuru and Wahala, 2003). Nevertheless, despite their wide scope, these procedures suffer from several drawbacks; some reagents are of limited stability, and preparation can be dangerous. Therefore, in this work we report a straightforward route for synthesis of a new estradiol derivative using $Cl_2/AlCl_3$:H_2O. The structure of estradiol derivative was confirmed using IR and NMR spectroscopy. The IR spectra contained characteristic vibrations at 3380 for hydroxyl groups; at 3330 for primary amino and 3310 for secondary amino. The ^{1}H NMR spectrum of the estradiol derivative shows signals at 0.88-2.55, 2.76 and 3.67 ppm for steroid nucleus. In addition, other signals at 0.76 ppm for methyl group; at 2.65, 2.79 and 3.74 ppm for arm bound to A ring of steroid nucleus. Finally, the spectrum contains a signal at 6.10 ppm for both hydroxyl and amine groups. The ^{13}C NMR spectra displays chemical shifts at 23.30-

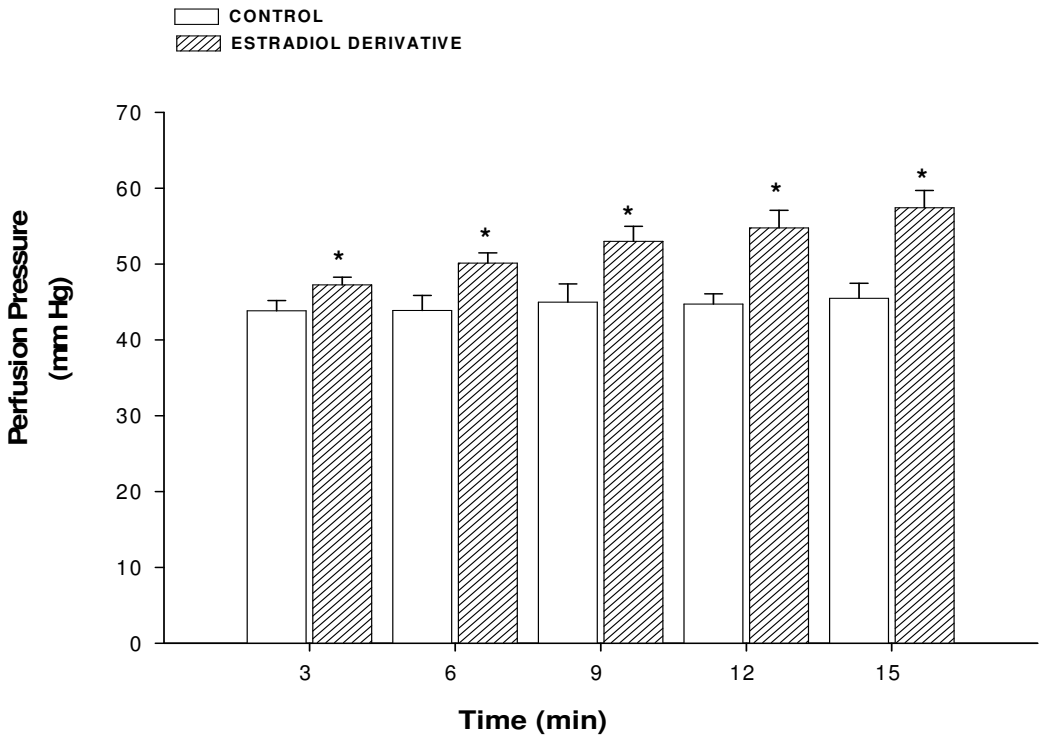

Figure 2. Effect induced by estradiol derivative on perfusion pressure. The results show that, estradiol derivative [10^{-9} mmol] significantly increase perfusion pressure (p = 0.005) through time (3 to 18 min) in comparison with the control conditions. Each bar represents the mean ± S.E. of 9 experiments.

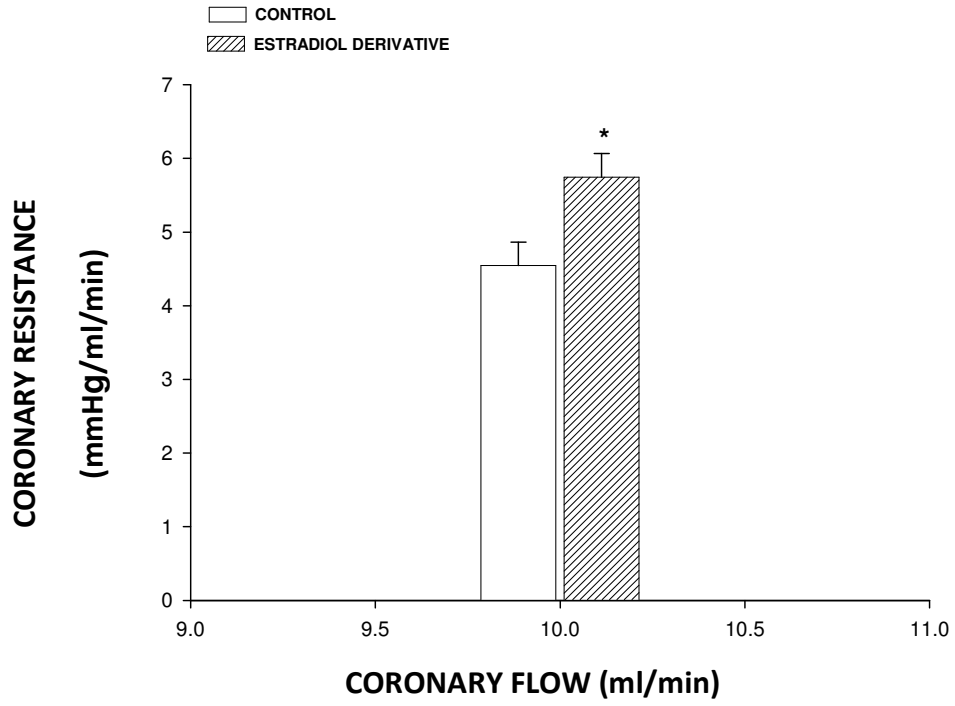

Figure 3. Activity exerted by estradiol derivative on coronary resistance. The results show that, coronary resistance was higher (p = 0.005) in the presence of estradiol derivative [10^{-9} mmol] in comparison with the control conditions. Each bar represents the mean ± S.E. of 9 experiments.

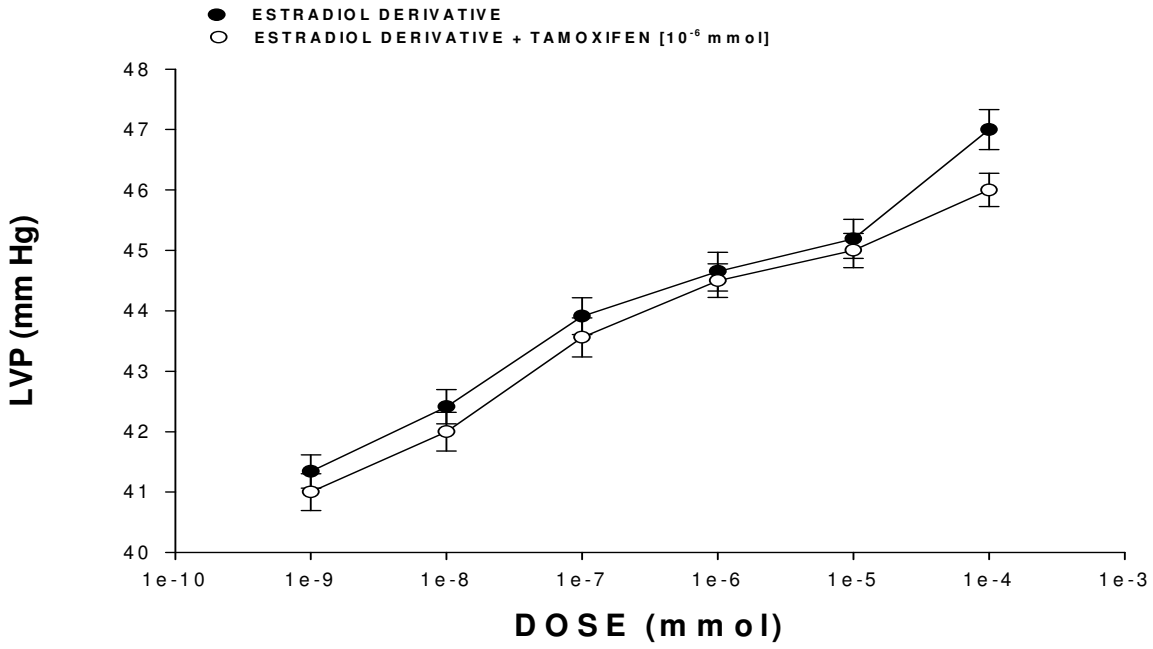

Figure 4. Effects induced by estradiol derivative on left ventricular pressure (LVP) through estrogen receptors. Intracoronary boluses (50 µl) of estradiol derivative [10^{-9} to 10^{-4} mmol]) were administered and the corresponding effect on the LVP was determined. The results showed that estradiol derivative increase the LVP in a dependent dose manner and this effect was not inhibited in presence of tamoxifen [10^{-6} mmol]. Each bar represents the mean ± S.E. of 9 experiments.

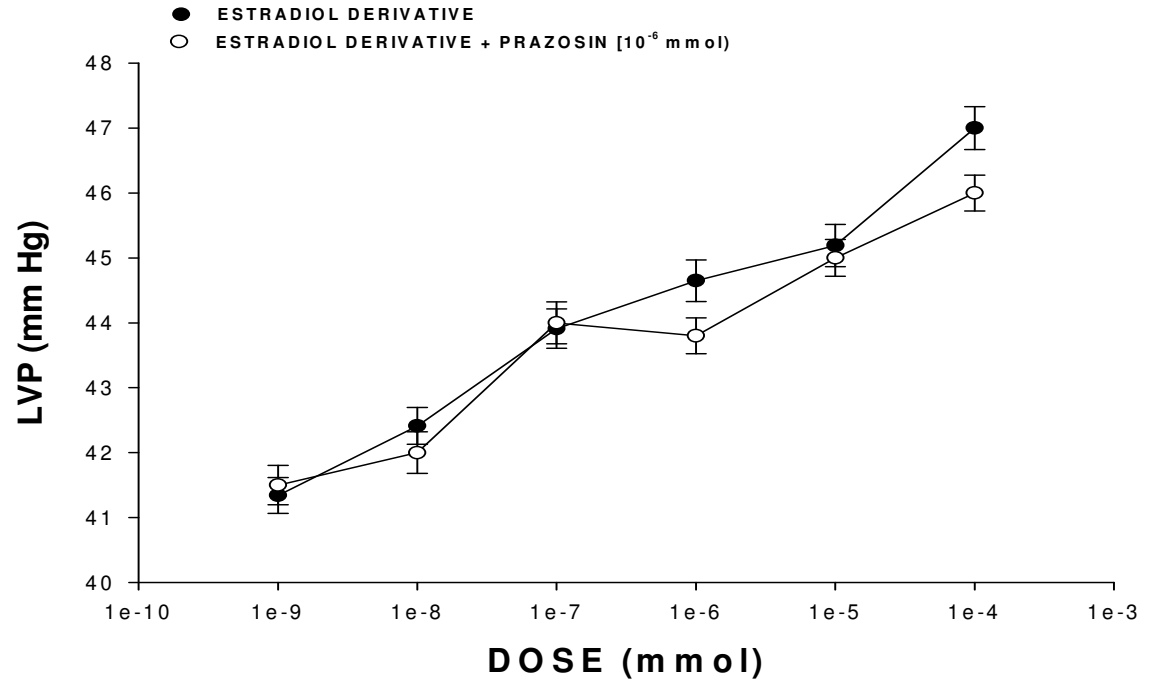

Figure 5. Effect exerted by estradiol derivative on LVP through of α1 adrenergic receptor. Estradiol derivative [10^{-9} to 10^{-4} mmol] was administered (intracoronary boluses, 50 µl) and the corresponding effect on the LVP was evaluated in absence and presence of prazosin [10^{-6} mmol]. The results showed that activity induced by estradiol derivative on LVP was not inhibited in presence of prazosin. Each bar represents the mean ± S.E. of 9 experiments.

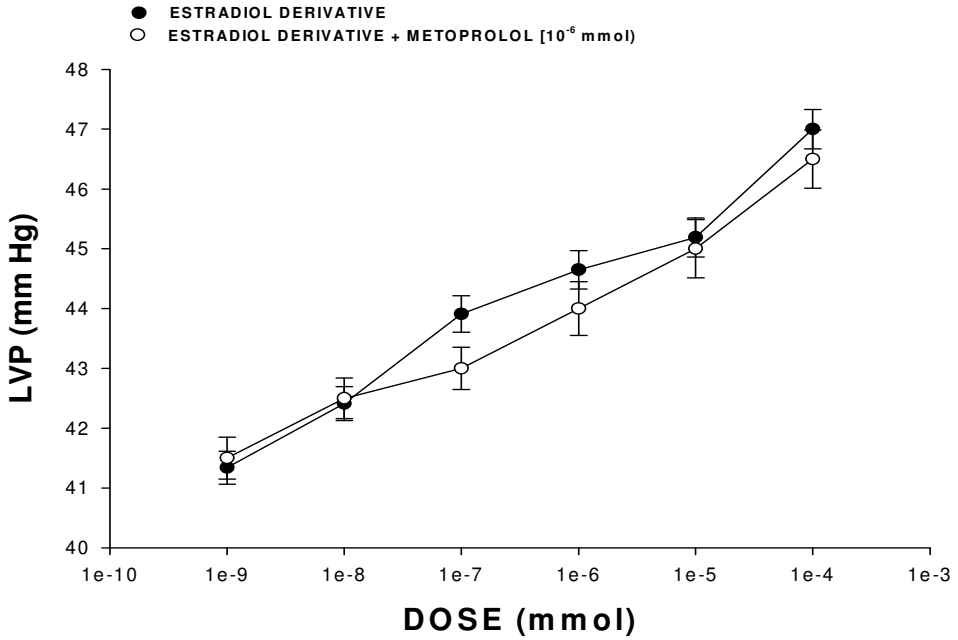

Figure 6. Activity induced by estradiol derivative on LVP through of β_1-adrenergic receptor. Intracoronary boluses (50 µl) of estradiol derivative [10^{-9} to 10^{-4} mmol] were administered and the corresponding effect on the perfusion pressure was evaluated in absence and presence of metoprolol (10^{-6} mmol). The results showed that, the activity induced by estradiol derivative on perfusion pressure was not inhibited in presence of metoprolol. Each bar represents the mean ± S.E. of 9 experiments.

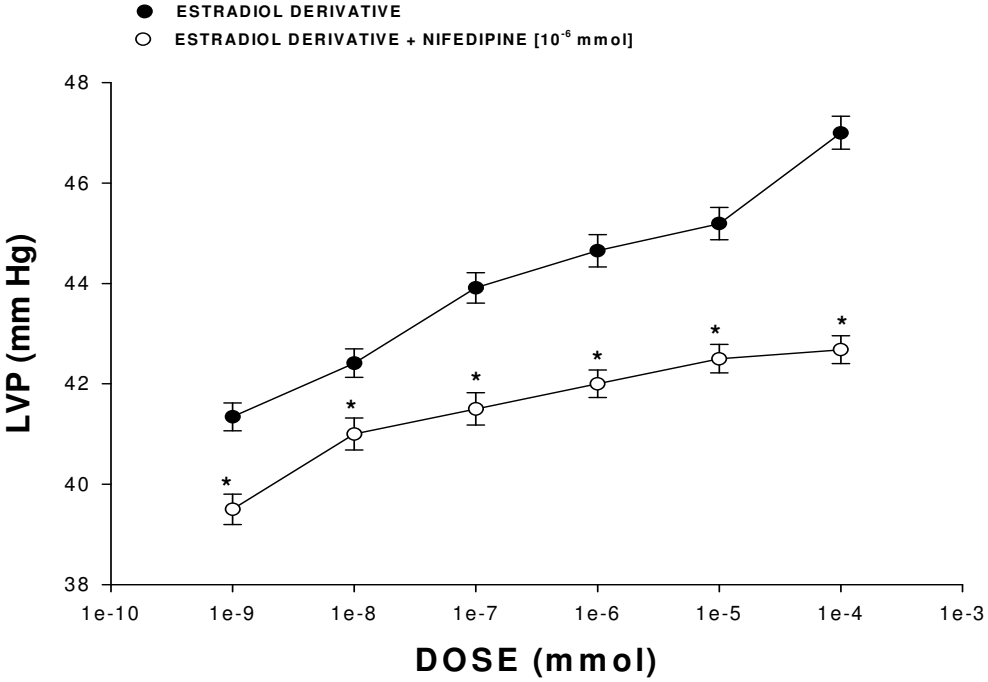

Figure 7. Activity exerted by estradiol derivative on perfusion pressure through L-type calcium channel. Intracoronary boluses (50 µl) of estradiol derivative [10^{-9} to 10^{-4} mmol] were administered in absence and presence of nifedipine [10^{-6} mmol]. The results showed that, the effect induced by estradiol derivative on perfusion pressure in presence of nifedipine was inhibited significantly (p = 0.005). Each bar represents the mean ± SE of 9 experiments.

Table 3. Effects induced by the estradiol derivative [10^{-9} mmol] on the intracellular calcium levels and absence or presence of nifedipine [10^{-6} mmol].

Ca_i^{++} [mM]	Time (min) E-D*	E-D+nifedipine**
3	1.35×10^{-3}	6.75×10^{-4}
6	1.35×10^{-3}	6.75×10^{-4}
9	1.35×10^{-3}	6.75×10^{-4}
12	2.03×10^{-3}	6.75×10^{-4}
15	2.03×10^{-3}	6.75×10^{-4}
18	2.03×10^{-3}	6.75×10^{-4}

*E-D (estradiol derivative); **$p = 0.005$.

38.43, 43.64-44.63, 50.53 and 81.66-145.30 ppm for steroid nucleus. In addition, several signals at 11.28 ppm for methyl group; at 41.38, 44.81 and 53.73 ppm for methylene groups involved in the arm bound to A ring of steroid nucleus. In addition, the presence of brucine derivative was further confirmed from mass spectrum which showed a molecular ion at m/z 376.30.

Biological evaluation

First stage

The activity induced by an estradiol derivative on the perfusion pressure and coronary resistance in isolated rat heart (Langendorff technique) was evaluated. The results obtained showed that, the estradiol derivative significantly increased the perfusion pressure in comparison with the control conditions and progesterone. Those experimental data indicate that, the estradiol derivative exerts effects on perfusion pressure, which could consequently bring modifications in coronary resistanceas happening in other type of steroid derivatives (Figueroa et al., 2009a; Figueroa-Valverde et al., 2010a, b). In order to verify this hypothesis, the effects induced by estradiol on coronary resistance were evaluated. The results indicate that coronary resistance in presence of estradiol derivative was higher in comparison with control conditions. All this data suggest that estradiol derivative may induce a positive inotropic activity in the isolated rat heart.

Second stage

In order to characterize the molecular mechanism of this phenomenon and analyzing the reports of Karas et al., (1994), which indicate that some estradiol derivatives exert its effect by activation of estrogen receptor in the vascular smooth muscle. For this reason, we used tamoxifen an estrogen receptor blocker to determine if the inotropic activity of estradiol derivative on left ventricular pressure was via the estrogen receptor which may be a key requirement for the biological activity as in the case of other estradiol derivatives (Haynes et al., 2000; Kaspar and Witzel, 1985). Our results showed that the effects of estradiol derivative were not inhibited by tamoxifen, suggesting that the molecular mechanism is not via the estrogen-receptor.

Analyzing this result and the reports of Colucci et al., (1982) suggests that estradiol exert an indirect tonic effect on adrenal catecholamines concentration (Lilley et al., 1976). To evaluate this hypothesis in this study, the effect exerted by the estradiol derivative on left ventricular pressure was evaluated in absence or presence of prazosin and metoprolol. The results showed that, the effect induced by the estradiol-derivative was not inhibited in presence of these compounds. These data indicate that the molecular mechanism involved in the effects of this steroid-derivative on left ventricular pressure is not through adrenergic activity.

Therefore, analyzing these results and other reports which suggest that activity induced by anestradiol-ethylenediamine conjugate on blood pressure involved a molecular mechanism via calcium-channels (Figueroa-Valverde et al., 2011d). In this work, the activity induced by the estradiol derivative on left ventricular pressure was evaluated in absence or presence of nifedipine. The results showed that effect exerted by progesterone-derivative was significantly inhibited in presence of nifedipine. These results suggest that, the effect of the estradiol derivative on left ventricular pressure may be to increase calcium levels through activation of the L-type calcium channel. Nevertheless, to evaluate this hypothesis and analyzing other reports which suggests that the effect induced by some steroid derivatives on left ventricular pressure involving increase in intracellular calcium (Ceballos et al., 1999; Figueoa-Valverde et al., 2009b). Therefore, in this study the activities induced by the estradiol derivative on intracellular calcium levels were evaluated in absence or presence of nifedipine. The results showed that, the effect exerted by the estradiol derivative induces increase in the intracellular calcium levels as a consequence of increases in the time; nevertheless, this effect is significantly reduced in

presence of nifedipine.

Conclusions

All these experimental data suggest that, inotropic activity positively induced byestradiolderivative on the left ventricular pressure may involve the L-type calcium channel activation.

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