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Activity exerted by a benzamide derivative on injury by ischemia/reperfusion in an isolated heart model

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Several studies indicate that some benzamide-derivatives have activity at cardiovascular level; nevertheless, there is scarce information about the effects exerted by the benzamide derivatives on cardiac injury caused by ischemia/reperfusion (I/R). In this experimental study, a new benzamide-derivative was synthetized with the objective of evaluating its activity on I/R in an I/R model of rat heart using the Langendorff technique. In addition, molecular mechanism involved in the effect induced by the benzamide derivative on perfusion pressure and coronary resistance was evaluated by measuring left ventricular pressure in the absence or presence of following compounds; nifedipine, indomethacin, propranolol and metoprolol. The results showed that the benzamide-derivative reduces infarct size compared with control. Other results showed that the benzamide derivative significantly increase the perfusion pressure and coronary resistance in isolated heart. Other data indicate that the benzamide-derivative increase left ventricular pressure in a dose-dependent manner (0.001 to 100 nM); however, this phenomenon was significantly inhibited by propranolol and metoprolol at a dose of 1 nM (p=0.05). In conclusion, these data suggest that cardioprotective activity of the benzamide-derivative is by stimulating catecholamine production and consequently induce changes in the left ventricular pressure levels. This phenomenon results in decrease of myocardial necrosis after ischemia and reperfusion.

Key words: Heart, benzamide derivative, ischemia, propranolol, metoprolol.

INTRODUCTION

Myocardial infarction is a major cause of death and disability worldwide (Yusuf et al., 2005; Thygesen et al., 2007); this cardiovascular disease is due to cell death of cardiac myocytes caused by ischaemia, which is the result of a perfusion imbalance between supply and demand. In addition, acute myocardial infarction can produce alterations in the topography of both the infarcted and noninfarcted regions of the ventricle (Pfeffer, 1995). There are some reports which indicate that the most effective method of limiting necrosis is the restoration of blood flow; however, the effects of reperfusion itself may also be associated with tissue injury (Klone et al., 1989). In this sense, there are studies which show that some drugs reduce myocardial necrosis in rabbits after ischemia and reperfusion (Hale et al., 1996). For example, astudy showed that rosiglitazone reduced myocardial infarction and improved contractile dysfunction caused by ischemia/reperfusion injury; nevertheless, the

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cardioprotective effect of rosiglitazone was most likely due to inhibition of the inflammatory response (Yue et al., 2001). Other data indicate that methylene blue can decrease injury by ischemia/reperfusion by reduction of molecular oxygen (Salaris et al., 1991). Also, other report showed that injury by ischemia/reperfusion is reduced with Levosidan via K_{ATP} Channels (Toit et al., 1999).

On the other hand, a report indicate that some benzamide derivatives also can exert effect on injury by ischemia/reperfusion; for example, a study showed that the compound N-(3,5-Bis-trifluoromethyl-phenyl)-5-chloro-2-hydroxy-benzamidecan decreasethe ischemia-reperfusion injury by inhibition of nuclear translocation of factor-kappa B (Onai et al., 2004). In addition, there are data which indicate that 3-aminobenzamide exert significant protective effects in myocardial reperfusion injury via activation of poly (ADP-ribose) synthetase which plays a role in the pathophysiology of acute myocardial infarction (Zingarelli et al., 1997). All these data show that several benzamide derivatives exert effects on the cardiovascular system; nevertheless, they do not show clearly the cellular site and actual molecular mechanisms of these compounds; therefore, data are needed for characterizing the activity induced by benzamide derivatives on ischemia-reperfusion injury. To test this aspect, the present experimental study was designed to investigate the effects induced by a benzamide derivative in a myocardial ischaemia/reperfusion model using Langendorff technique. In addition, it was thought desirable to evaluate the molecular mechanism involved in the activity of the benzamide derivative on left ventricular pressure, using some pharmacological tools for blocking various biological systems such as nifedipine [calcium channel antagonist] (Henry, 1980), indomethacin [prostanglandin synthesis blocker] (Owen et al., 1975), propranolol [β_1 receptor blocker] (Sklar et al., 1982), metoprolol [selective β_1 receptor antagonist] (Bengtsson et al., 1975).

MATERIALS AND METHODS

Chemical synthesis

Compound 1 (1-[(2-Amino-ethylamino)-phenyl-methyl]-naphthalen-2-ol) was prepared according to a previously reported method (Figueroa-Valverde et al., 2013). The other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. The melting point for the danazol derivative was 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 (nuclear magnetic resonance) spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75.4 MHz in $CDCl_3$ (deuterated chloform) using tetramethylsilane (TMS) as internal standard. Electron impact mass spectroscopy (EIMS) spectra were obtained with a Finnigan Trace Gas Chromatography Polaris Q Spectrometer. Elementary analysis data were acquired from a Perkin Elmer Ser. II CHNS/0 2400 elemental analyzer.

Synthesis of 2,4-dinitro-N-(2-{[(1E)-phenylmethylene]amino}ethyl)benzamide (compound 3)

A solution of compound 1 (100 mg, 0.29 mmol), benzaldehyde (50 mg, 0.29 mmol) and boric acid in 10 ml of methanol was stirred for 24 h at room temperature. The reaction mixture was evaporated to dryness under reduced pressure, the residue was washed 3 times with water. Then, the precipitate was separated and dried at room temperature.

Biological method

All experimental procedures and protocols 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 (Bayne, 1996). Male Wistar rats weighing 200 to 250 g were obtained from University Autonomous of Campeche.

Reagents

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

Experimental design

Briefly, male rat (200 to 250 g) was anesthetized by injecting them with pentobarbitalat 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. The 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 (95:5/5%). 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 15 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

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 (LV/dP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium (Figueroa-Valverde et al., 2011a). The latex balloon was bound to cannula which was linked to pressure transducer that was connected with the MP100 data acquisition system.

First stage

Ischemia/Reperfusion model: After of 15 min equilibration time, the hearts were subjected to ischemia for 30 min by turning off the perfusion system (Booth et al., 2005). After this period, the system was restarted and the hearts were reperfused 30 min with Krebs-Henseleit solution. The hearts were randomly divided into 2 major

treatment groups with n = 9: Group I, hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution); Group II, hearts were subjected to ischemia/reperfusion and treated with benzamide derivative (0.001 nM) before ischemia period (for 10 min) 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 side armport connected to the aortic cannula. The dye was passed through the heart for 10 s to ensure its uniform tissue distribution. The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region. 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.The areas of the normal left ventricle non risk region, area at risk, and infarct region were determined using the technique reported by Boot et al. (2005). Total area at risk was expressed as the percentage of the left ventricle.

Second stage

Effect induced by the benzamide derivative on perfusion pressure: Changes in perfusion pressure as a consequence of increases in time (3 to 18 min) in absence (control) or presence of the benzamide derivative at a concentration of 0.001 nM 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 benzamide derivative on coronaryresistance: The coronary resistance in absence (control) or presence of the benzamide derivative at a concentration of 0.001 nM 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 reflected the changes in coronary resistance.

Third stage

Effects of the benzamide derivative on left ventricular pressure through the calcium channel: Intra coronary boluses (50 µl) of the benzamide 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 aperiod of 10 min).

Effect exerted by the benzamide derivative on leftventricular pressure in the presence of indomethacin: The boluses (50 µl) of the danazol derivative [0.001 to 100 nM] were administered and the corresponding effect on the left ventricular pressure was evaluated. The bolus injection administered was done in the point of cannulation. The dose response 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 benzamide derivative on left ventricular pressure through β_1- adrenergic receptor: Intracoronary boluses (50 µl) of the benzamide derivative (0.001 to 100 nM) were administered and the corresponding effect on the left ventricular pressure was determined. The dose-response curve (control) was repeated in the presence of propranolol or metoprolol at a concentration of 1 nM (duration of preincubation with propanolol or metoprolol was by a 10 min equilibration period).

Statistical analysis

The obtained values are expressed as average ± standard error (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 (Hocht et al., 1999). The differences were considered significant when p was equal or smaller than 0.05.

RESULTS

Chemical synthesis

The yield of the reaction product (compound 3, Figure 1) was 70% with melting point of 306 to 308°C. In addition, the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) at 3320, 1638 and 1350. In addition, the chemical shifts of the spectroscopic analyses of ^{1}H NMR and ^{13}C NMR for the benzamide derivative are shown in Tables 1. Finally, the results of mass spectroscopy (MS) (70 eV) was shown as m/z 342.02. Additionally, the elementary analysis data for the benzamide derivative ($C_{16}H_{14}N_4O_5$) were calculated (C, 56.14; H, 4.12; N, 16.37; O, 23.37) and found (C, 56.10; H, 4.09).

Biological activity

First stage

Effect of benzamide derivative on ischemia/reperfusion injury: The results (Figures 2 and 3) showed that the benzamide derivative reduces infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts (control).

Second stage

In this study, the activity induced by the benzamide derivative on perfusion pressure and coronary resistance in the isolated rat hearts was evaluated. The results obtained from changes in perfusion pressure as a consequence of increases in the time (3 to18 min) in absence (control) or in presence of benzamide derivative (Figure 4), showed that benzamide derivative [1 nM] significantly increase the perfusion pressure (p=0.05) in comparison with the control conditions [1 nM]. In addition, another result (Figure 5) showed that coronary resistance, calculated as the ratio of perfusion pressure at coronary flow assayed (10 ml/min) was higher in the presence of the benzamide derivative in comparison with the control conditions (p=0.05) at a concentration of 1 nM.

Third stage

Other results showed that activity exerted by the benzamide derivative [0.001 to 100 nM] increased the left ventricular pressure and this effect was not inhibited in

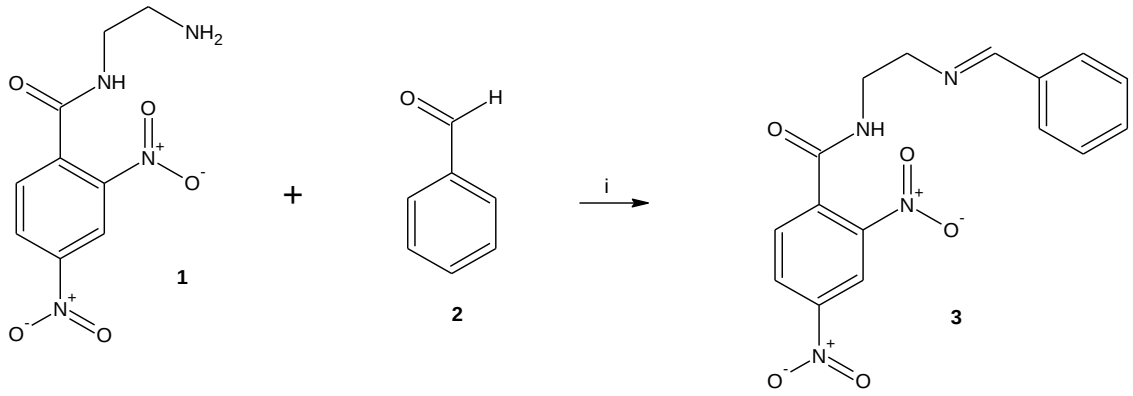

Figure 1. Synthesis of 2,4-dinitro-*N*-(2-{[(1*E*)-phenylmethylene]amino}ethyl)benzamide (**3**). Reaction of *N*-(2-aminoethyl)-2,4-dinitrobenzamide (**1**) with benzaldehyde (**2**) to form compound **3**. i = boric acid.

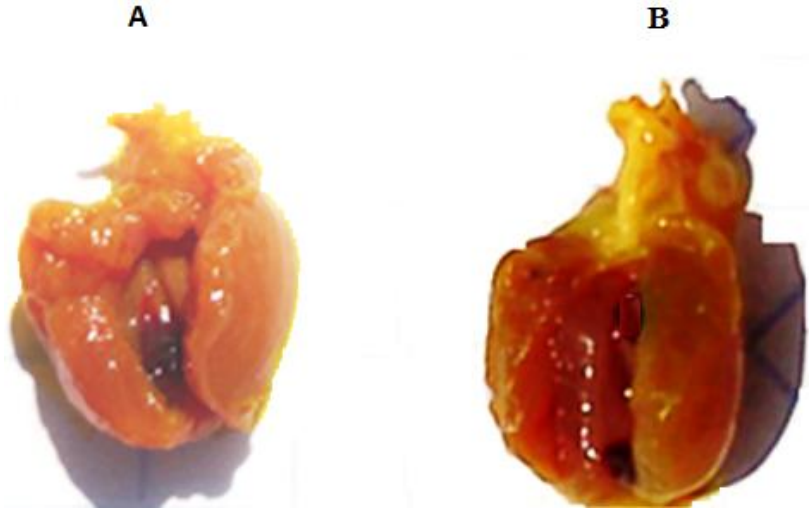

Figure 2. Comparison of cardioprotective effect of the benzamide derivative (**B**) at a dose of 1 nM with the control (**A**) on the functional recovery of rat hearts subjected to ischemia and reperfusion.

presence of nifedipine or indomethacin drugs (Figure 6 and 7) at a concentration of 1 nM. Finally, other data obtained (Figure 8) indicate that the benzamide derivative induces an increase in left ventricular pressure in a dose dependent manner [0.001 to 100 nM] and this effect was significantly inhibited by propranolol ($p = 0.05$) and metoprolol ($p = 0.05$) at a dose of 1 nM.

DISCUSSION

Chemical synthesis

In this study, a straight forward route for the preparation of (2,4-dinitro-*N*-(2-{[(1*E*)-phenylmethylene]amino}ethyl)benzamide (compound 3) was reported. The synthesis was achieved by the formation of an imine group (Schiff base) involved in compound 3 (Figure 1). There are several procedures for the synthesis of imines which are described in the literature (Shirayev et al., 2005; Uppiah et al., 2009; Figueroa-Valverde et al., 2012). In this study, the synthesis of the compound 3 was developed by the reaction of *N*-(2-aminoethyl)-2,4-dinitrobenzamide with benzaldehyde using boric acid as catalyst to form the compound 3. The structure of the benzamide derivative was confirmed using IR and NMR spectroscopy (Tables 1 and 2). The IR spectra contained characteristic vibrations at 33200 for imino group; at 1638 for amide group and 1350 for nitro groups. The ^{1}H NMR spectrum of the benzamide derivative shows signals at 3.72 to 3.75 ppm for arm bound to both imino and amino groups; at 7.38 to 7.68 ppm for protons involved in the phenyl group which is bound to imino group; at 7.83 ppm for amide group; at

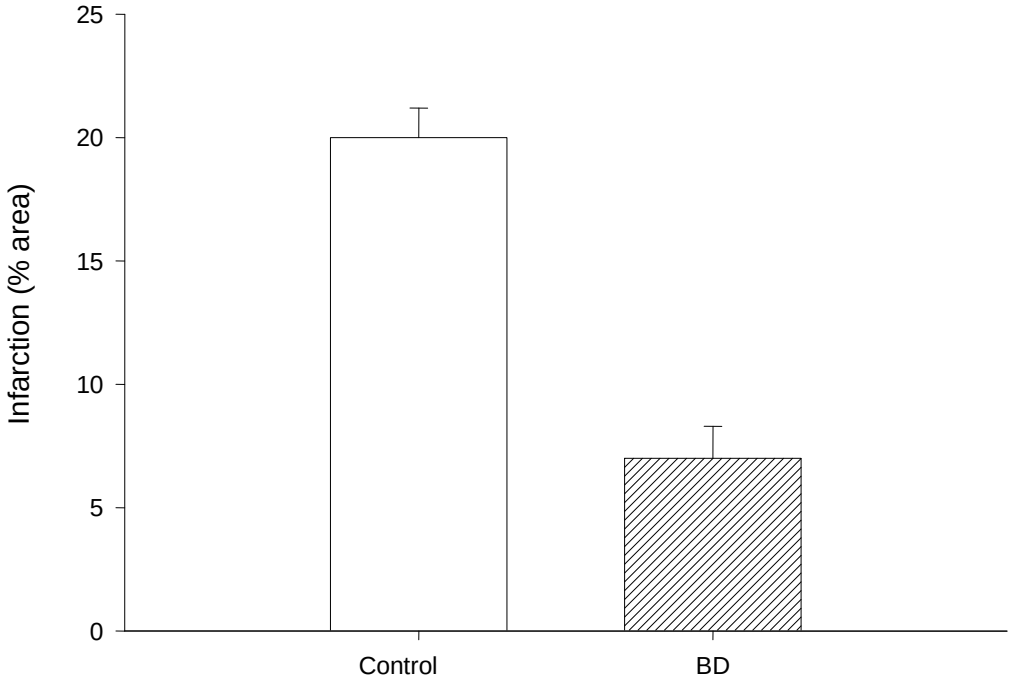

Figure 3. Effect exerted by BD on cardiac ischemia/reperfusion with the control. The results showed that BD significantly reduced infarct size expressed as a percentage of the area at risk compared with the vehicle-treated hearts (p = 0.05). Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative.

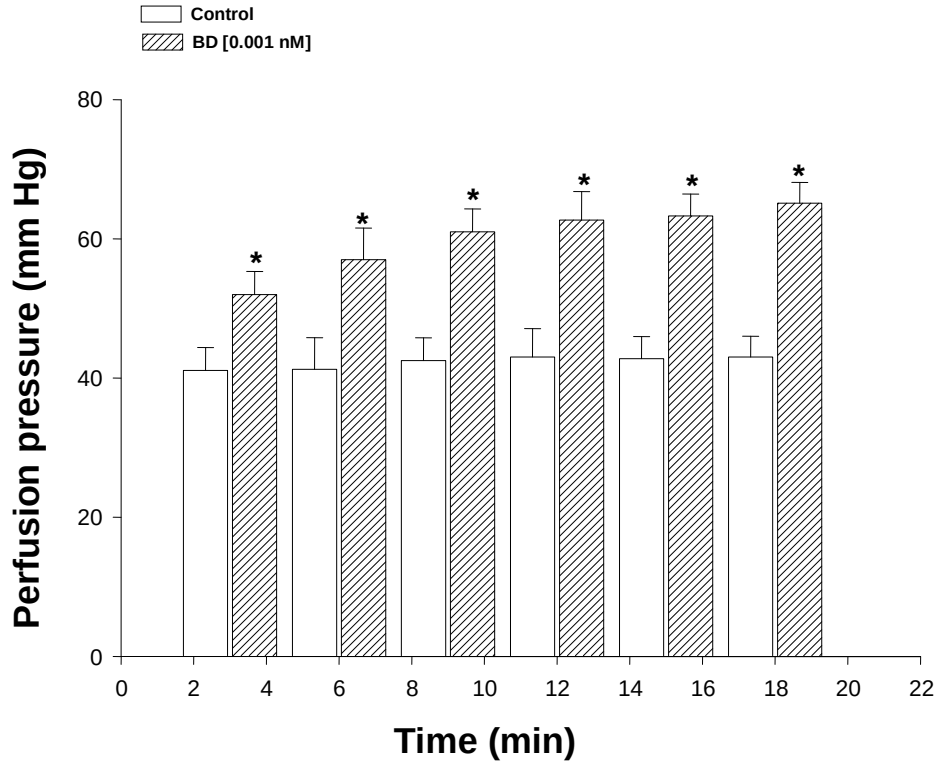

Figure 4. Effect induced by BD on perfusion pressure. The results show that BD significantly increase perfusion pressure (**p** = 0.05) through time in comparison with the control conditions. Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative.

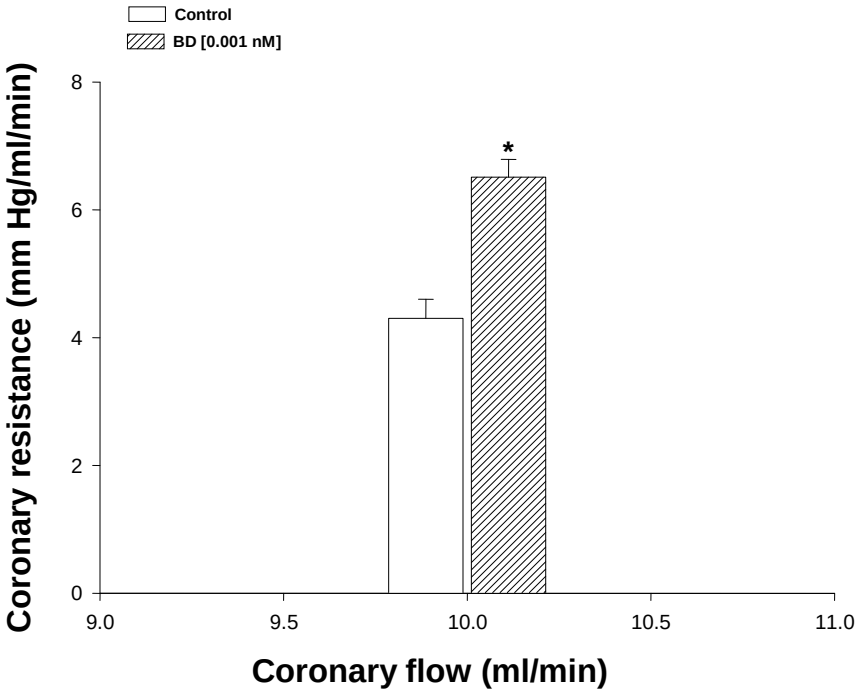

Figure 5. Activity exerted by BD on coronary resistance. The results show that coronary resistance was higher (**p** = 0.06) in the presence of BD in comparison with the control conditions. Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative.

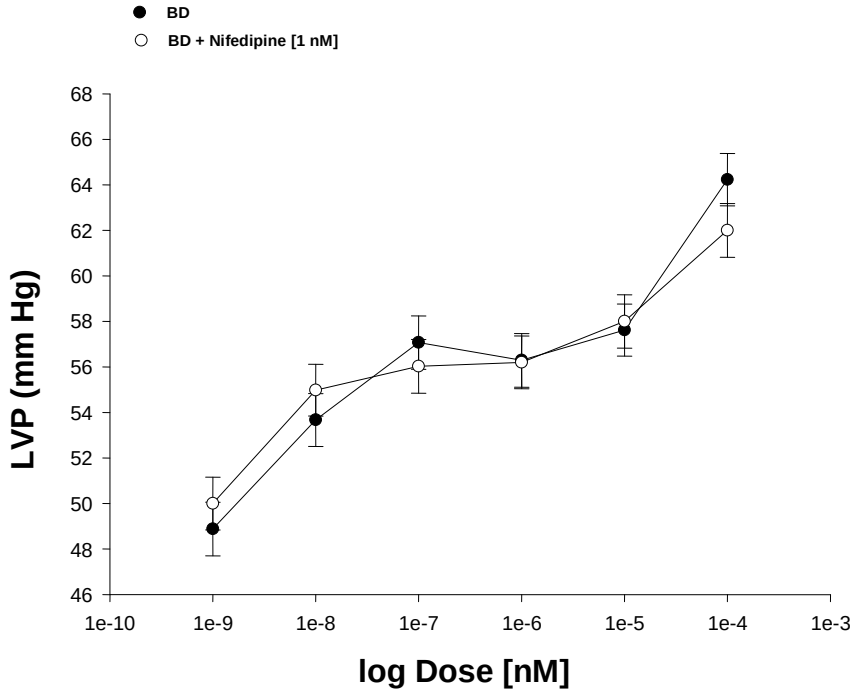

Figure 6. Effects induced by BD on LVP through calcium channel activation. Intracoronary boluses (50 µl) of BD [0.001 to 100 nM] were administered and the corresponding effect on the LVP was determined. The results showed that BD increase the LVP in a dependent dose manner and this effect was not inhibited in the presence of nifedipine. Each bar represents the mean ± SE of 9 experiments. BD = benzamide derivative; LVO = left ventricular pressure.

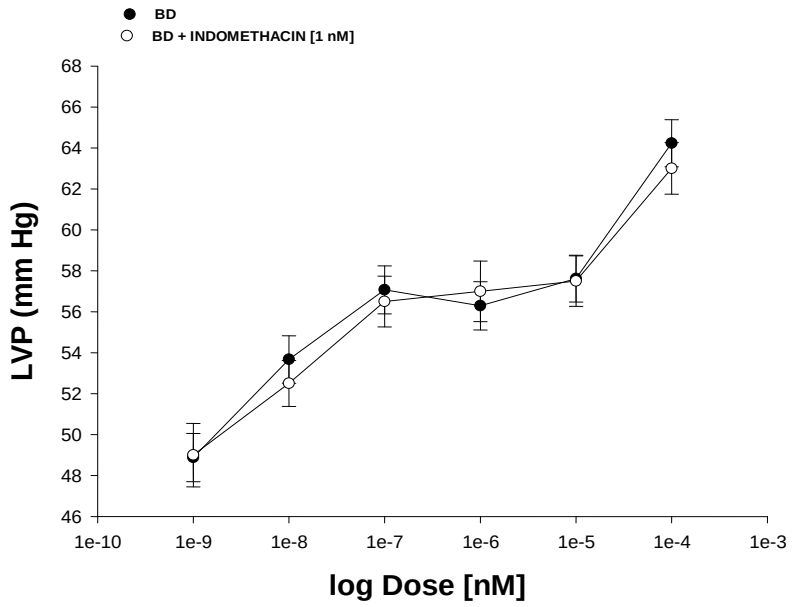

Figure 7. Effects induced by BD on LVP through prostaglandins synthesis. Intracoronary boluses (50 µl) of BD [0.001 to 100 nM] were administered and the corresponding effect on the LVP was determined. The results showed that BD increase the LVP in a dependent dose manner and this effect was not inhibited in the presence of indomethacin. Each bar represents the mean ± S.E. of 9 experiments. BD = benzamide derivative; LVO = left ventricular pressure.

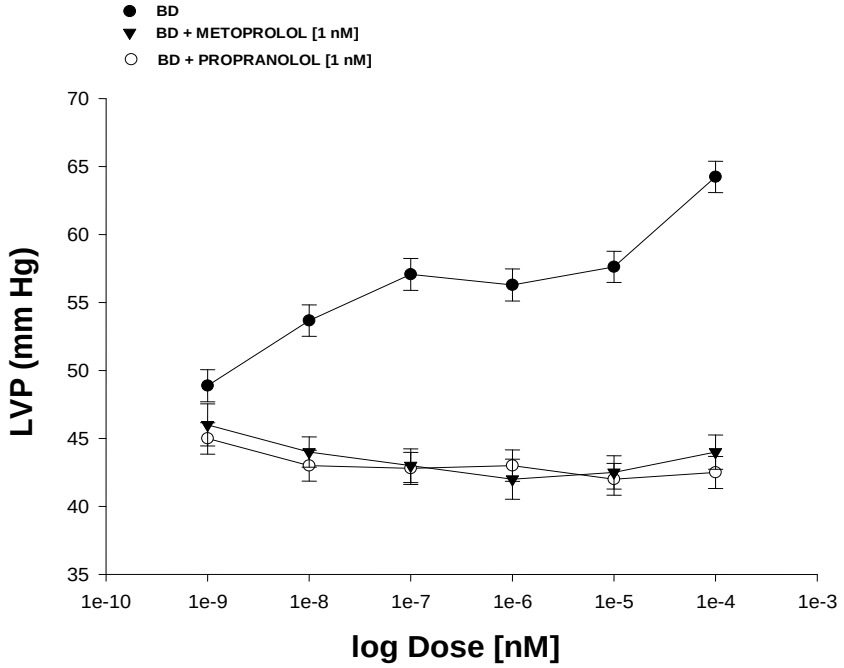

Figure 8. Activity exerted by BD on LVP through of β-adrenergic receptors.BD [0.001 to 100 nM] was administered (intracoronary boluses, 50 µl) and the corresponding effect on the LVP was evaluated in the absence and presence of propranolol or metoprolol. The results showed that activity induced by BD on LVP was significantly inhibited in the presence of propranolol ($p = 0.05$) or metoprolol ($p = 0.05$). Each bar represents the mean ± S.E. of 9 experiments. BD = benzamide derivative; LVO = left ventricular pressure.BD = benzamide derivative; LVO = left ventricular pressure.

Table 1. Analysis of spectroscopicdata (300 MHz, $CDCl_3$).

Parameter	^{1}H NMR(ppm)	^{3}C NMR (ppm)
Benzamide derivative	3.72 (m, 2H)	35.78 (**C**-16)
	3.75 (m, 2H)	62.59 (**C**-17)
	7.38-7.68 (m, 5H)	120.91 (**C**-2)
	7.83 (broad, 1H)	128.04 (**C**-4)
	8.24 (d, 1H, *J* = 1.20)	128.18 (**C**-5)
	8.33-8.81 (d, 3H, *J* = 8.24)	128.20 (**C**-21,**C**-25)
	-	129.28 (**C**-22, **C**-24)
	-	130.62 (**C**-23)
	-	136.66 (**C**-20)
	-	141.57 (**C**-6)
	-	143.43 (**C**-1)
	-	147.50 (**C**-3)
	-	156.96 (**C**-19)
	-	163.77 (**C**-11)

8.24 ppm for methylene group bound to both amino and phenyl groups; at 8.33 to 8.81 ppm for protons involved in phenyl group bound to both nitro groups. The ^{13}C NMR spectra displays chemical shifts at 35.78 to 62.59 ppm for arm bound to both imino and amino groups; at 120.91 to 128.18 ppm for protons involved in phenyl group which is bound to both nitro groups; at 128.20 to 136.66 ppm for carbons of phenyl group bound to imino group; at 141.57 to 147.50 ppm for carbons involved in phenyl group bound to nitro groups. Finally, other signals at 156.96 ppm for methylene group bound to both imino and phenyl groups; at 163.77 ppm for amide group were found. In addition, the presence of benzamide derivative was further confirmed from mass spectrum which showed a molecular ion at m/z 342.02.

Biological evaluation

In this study, the activity of benzamide derivative was evaluated in an ischemia-reperfusion model. The results showed that benzamide derivative reduced infarct size expressed as a percentage of the area at risk compared with vehicle-treated hearts (control). This phenomenon can be conditioned by activation of some structure biological (that is, ionic channels or specific receptors) involved in the endothelium of coronary artery (Bouis et al., 2009) or by the influence exerted by benzamide derivative on blood pressure which consequently bring reduction in the infarct size, and decrease the myocardial injury after ischemia-reperfusion similar to other reports for other compounds such as estrogens (Beer et al., 2002). In order to evaluate this hypothesis, the effect exerted by the benzamide derivative on blood vessel capacity and coronary resistance, translated as changes in perfusion pressure was evaluated in an isolated rat heart model. The results show that the benzamide derivative significantly increases the perfusion pressure over time (3 to 18 min) compared to the control conditions. These data suggest that the benzamide derivative exerts effects on perfusion pressure which could subsequently modify vascular tone and coronary resistance of heart. Therefore, in this study, the activity exerted by the benzamide derivative on coronary resistance was evaluated. The results indicate that coronary resistance was increased in the presence of this compound. These data suggest that the benzamide derivative exerts effect on vascular tone through of generation or activation of vasoactive substances such as intracellular calcium happening with other type of compounds as the carbamazepine-alkyne derivative (Figueroa-Valverde et al., 2011a).

In order to characterize the molecular mechanism of this phenomenon and analyzed the reports of some investigations which indicate that some steroid derivatives induces its effect on blood pressure via the calcium channels activation (Figueroa-Valverde et al., 2011b). In addition, a report showed that some positive cardiotonic agents act by an increase in intracellular Ca^{2+} and consequently induce an increase in the sensitivity of contractile proteins to Ca^{2+} ions or by combinations of the two mechanisms (Bowman et al., 1999). Therefore, in this study, the activity induced by the benzamide deri-vative on left ventricular pressure was evaluated in the absence or presence of nifedipine. The results showed that effect exerted by the benzamide derivative was not inhibited in the presence of nifedipine. Furthermore, these data indicate that activity exerted by the benzamide derivative was not via activation calcium channel.

Analyzing experimental data obtained, validating the effect induced by some steroid derivatives on perfusion pressure via prostanglandins synthesis was also considered (Sheillan et al., 1983) and to evaluate the possibility that the activities exerted by the benzamide derivative involve stimulation and secretion of prostaglandins.

In this sense, in this experimental study,the activity exerted by the benzamide derivative on left ventricular pressure in the absence or presence of indomethacin was evaluated. The results showed that effect induced by the benzamide derivative on left ventricular pressure was not blocked by indomethacin. These results indicate that the molecular mechanism involved in the effect exerted by the benzamide derivative was not via prostaglandins.

Moreover, in the search of the molecular mechanism involved in activity induced by the benzamide derivative on left ventricular pressure and analyzing previous reports, which indicate that some substances such as progesterone can stimulate catecholamine (Tollan et al., 1993) which has an important role in the development or maintenance of elevated blood pressure (Lilley et al., 1976); in this study, the effect exerted by the benzamide derivative on left ventricular pressure was evaluated in the absence or presence of propranolol or metoprolol. The results showed that the effect induced by the benzamide derivative was significantly inhibited in the presence of this compound. All these data suggest that the molecular mechanism involved in the activity of benzamide derivative is via adrenergic system. This phenomenon is similar to activity exerted by other drugs on left ventricular pressure (Thiemermann et al., 1997) which may contribute to decrease cell death caused by ischemia/reperfusion in men.

Conclusion

The benzamide derivative is a particularly interesting drug, because the activity induced 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.

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