Original Article

Functional and Morphological Study of the Effects of Carvacrol on Smooth Muscle of the Thoracic Aorta in the Rat

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Abstract

Background: The leaves of *Origanum* are widely used in herbal medicine hence of having many beneficial ingredients, one of these important compounds is Carvacrol. The inhibitory effect of Carvacrol was the core of this study by applying different kinds of stimulants to smooth muscles in the wall of thoracic aorta in rats. **Aim:** To investigate the pharmacological effects of Carvacrol, the main active ingredient present in the medicinal plant Origanum, on the contractile activity and morphology of the smooth muscle of the rat thoracic aorta. **Materials and Methods:** After the thoracic aorta arteries were isolated and prepared for the experiments, each thoracic aorta was cut into 5-mm ring segments; different stimulants were used (Potassium Chloride, Norepinephrine, U46619, and α,β-methylene ATP) in the presence and absence of Carvacrol on four groups of rats. The isolated rings were placed and connected to a force transducer which in turn linked to a data acquisition system via an amplifier to record the effect of each stimulant. GraphPad Prism version 5.02 for Windows, one-way analysis of variance followed by Dunnett's multiple comparison test. **Results:** It was found out that Carvacrol obstructs the contractile responses elicited by exogenous NA, KCl, U46619, and α,β-methylene ATP in a concentration dependent manner. **Conclusion:** The addition of Carvacrol in the experimental rats showed an increase in the thickness of tunica media as evident by the number of smooth muscle layers and laminae of elastic fibers. It was found that Carvacrol reduced the vascular smooth muscle contractility in the rat thoracic aorta. The mechanism of action is presumed to be achieved through interfering with the mobilization of both intracellular and extracellular $Ca2^{+}$ through different receptors. Furthermore, it might be suggested that Carvacrol in high doses stimulates smooth muscles in the wall of aorta leading to an increase in the thickness of tunica media layer.

Keywords: *Carvacrol, Origanum, thoracic aorta, vascular smooth muscle*

Introduction

The use of herbal medicinal remedies is an integral part in the traditional medicine of some communities in worldwide;[1] hence, herbs are available, easy to be prepared for oral consumption, and they are relatively of low cost. Nearly 10% of the total flowering plants are classified as herbs.[2] In fact, medicinal plants have been used to treat disorders of gastrointestinal tract,[3] respiratory system,[4] cardiovascular diseases,[5] and diabetes.[6]

Origanum (Family Labiatae) is a subtropical herb that has been cultivated in the Mediterranean region. The Origanum essential oils are composed of more than 20 ingredients including

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Carvacrol (5-isopropyl-2-methylphenol), p-Cymene, γ Terpinene, Linalool, Cadinol, Cadinene, and Thymol. Origanum has numerous species such as *Origanum vulgare, Origanum onites, Origanum syriacum, Origanum majorana, Origanum compactum, Origanum microphyllum, and Origanum acutidens*.[7] It was found out that Carvacrol existed in high amounts in essential oils which can most probably explain the pharmacological actions of essential oils. In addition to that, many studies have shown that Carvacrol has many pharmacological and biological properties. For example, Carvacrol has shown antioxidant,[8] antibacterial,[9] anticancerous,[10] anti-inflammatory,[11] and vasorelaxant features.[12,13]

The aim of this study was to investigate the possible inhibitory effect of the active ingredients of Origanum, especially the Carvacrol ingredient on the smooth muscles of an isolated rat thoracic aorta using not only NA and KCl as stimulants but also ATP under possible different conditions of vascular tone. The combination of morphological experiments in the present study may help to fill the gap in literature about the direct effects of Carvacrol on the ultrastructure of vascular smooth muscle.

Materials and Methods

Animals

Sixteen male Wister rats (250-300 g and 5-7 weeks aged) were obtained from the animal colony of the School of Medicine at The University of Jordan. Before experiments, animals were housed under controlled conditions of temperature (21 ± 1 C̊), exposed to daily 12 h light dark cycle, and had free access to food and water. All animal experiments were conducted in concordance with the University of Jordan's "Regulations and Ethical Guidelines for the Care and Use of Laboratory Animals."

Drugs

Carvacrol, Noradrenaline Hydrochloride, Potassium Chloride, U46619, and α,β-methylene ATP were obtained from SIGMA- ALDRICH, USA. Carvacrol was dissolved in Dimethyl Sulfoxide (DMSO) and stored at room temperature; all other drugs were dissolved in water and stored at -20°C.

Tissue preparation

On the day of experiments, rat was stunned by a blow to the cranium and killed by cervical dislocation. The thoracic cavity was opened and directly after the end of the arch of the aorta curve the thoracic aorta was identified which is the direct continuation of the arch of aorta in the thorax region above the diaphragm, then isolated, dissected, and cleaned from the surrounding connective tissue. The thoracic aorta was cut into 5-mm ring segments. The rings were transferred to 15-mL organ-bath filled with Krebs–Henseleit solution containing the following (mM): NaCl, 136.8; KCl, 3.58; $MgSO_4$, 1.44; $KH2PO_4$, 1.63; $NaHCO_3$, 25; $CaCl_2$, 1.39; and glucose, 10. The solution was tested for pH = 7.4, maintained at 37°C, and gassed with a mixture of 95% O_2 and 5% CO_2. The isolated rings were placed between two stainless steel triangular hooks. The upper hook was connected by a thin thread to a force transducer (World Precision Instruments, Sarasota, Florida, USA) linked to a Maclab data acquisition system (AD Instruments Ltd, Hasting, UK) via an amplifier, while the lower hook was fixed on the tissue holder. The isolated thoracic aortic rings were then suspended between the two hooks under 2-g tension. Tension was measured by an isometric force transducer and recorded on a laboratory chart recorder.

After an equilibration period of an hour, the isolated rings were twice contracted by adding (60 mM) of Potassium Chloride (KCl) to the bath. After 30-min interval between the contractions, the isolated rings were then washed with Krebs–Henseleit solution and allowed to equilibrate for 60 min. Responses to a single application of NA (1 μM), a Thromboxane mimetic 9,11-dideoxy-11 alpha, 9 alpha epoxy methanoprostaglandin F2alpha U46619 (5-25 nM), KCl (60 mM), and α,β-methylene ATP (1 μM) were obtained in the presence and in the absence of different concentrations of Carvacrol (0.01, 0.05, 0.1, 0.5, 1, 10, and 20 mg/mL), respectively, beside these experiments further one performed by eliciting a contractions by application of NA (1 μM), then adding the DMSO solution without Carvacrol in a way to justify the effect of Carvacrol and to insulate the effect of DMSO itself.

Evaluation of the contractile responses of rat thoracic aorta evoked by noradrenaline

After a 60-min equilibration period, response to a single application of NA (1 μM) was conducted under basal tone conditions. The tissue was then washed twice with Krebs-Henseleit buffer and five subsequent applications of NA (1 μM) were conducted. After each application, the tissue was washed twice with Krebs-Henseleit buffer and left for 20 min. In a different experiment, the first single application of NA (1 μM) was recorded as control. The tissue was then washed twice and was incubated in ascending concentrations of Carvacrol (0.01, 0.05, 0.1, 0.5, 1, 10, and 20 mg/mL, respectively), with 20-min incubation period between each application

of NA (1 μM); beside these experiments further one performed by eliciting a contractions by application of NA (1 μM), then adding the DMSO solution without Carvacrol in a way to justify the effect of Carvacrol and to insulate the effect of DMSO itself.

Evaluation of the contractile responses of rat thoracic aorta evoked by potassium chloride

After a 60-min equilibration period, response to a single application of KCl (60 mM) was conducted under basal tone conditions. The tissue was then washed twice with Krebs-Henseleit buffer and five subsequent applications of KCl (60 mM) were conducted. After each application, the tissue was washed twice with Krebs-Henseleit buffer and left for 20 min.

In some experiments, a single application of KCl (60 mM) was added to the tissue and the contractile responses were recorded as control. The tissue was then washed twice with Krebs-Henseleit buffer and was exposed to Carvacrol (1, 10, and 20 mg/mL, respectively), with 20-min incubation period between each application.

Characterization of the contractile responses of rat thoracic aorta evoked by α,β-methyleneatp under raised tone conditions

After a 60-min equilibration period, a single application of α,β-methylene ATP (1 μM) under basal tone conditions was carried out; the tissue was then washed twice with Krebs-Henseleit buffer and allowed to equilibrate for 30 min. U46619 (5-25 nM) was then added to raise the tone to a level of about 25%-35% of contraction relative to the second KCl response. After raising the tone, a second application of α,β-methylene ATP (1 μM) was obtained.

Characterization of the contractile responses of rat thoracic aorta evoked by U46619

After a 60-min equilibration period, responses to accumulative addition of U46619 (5-25 nM) were recorded. In some groups, responses to U46619 (5-25 nM) were recorded as control. The tissue was then washed twice and another accumulative response to U46619 (5-25 nM) was built up. After reaching a plateau, Carvacrol (0.01, 0.1, 10, and 20 mg/mL) was added to the tissue.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). Results are expressed as the mean ± SEM. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Student's paired *t*-test if the data were normally distributed (checked by Shapiro-Wilk normality test), while Mann-Whitney test was used if the data were not normally distributed. A value of $P < .05$ was taken to indicate statistical significance.

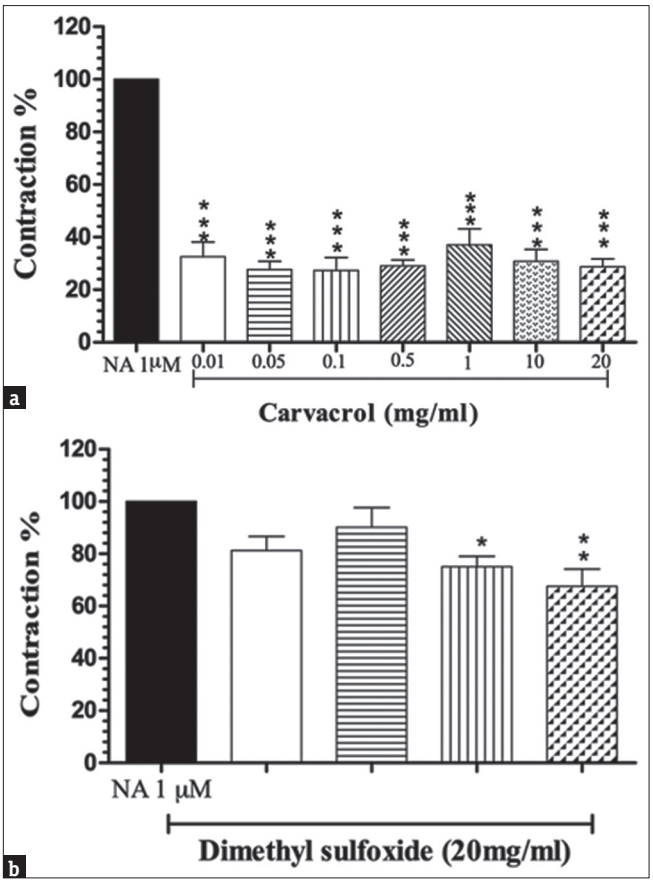

Figure 1: (a) Effects of Carvacrol on the contractile responses evoked by NA in rat aortic preparation (n = 15). (b) Effect of DMSO on contractile responses to Noradrenaline (1 μM), Each bar represents the mean ± SEM. ***P <.0001 vs. control (one way ANOVA followed by Dunnett's test)

Results

Effects of carvacrol on the vasocontractile responses evoked by noradrenline in rat thoracic aorta

A single application of NA (1 μM) evoked a contraction of 100% (n = 15). [Figure 1a] illustrates that these contractile responses were inhibited in the presence of Carvacrol (0.01, 0.05, 0.1, 0.5, 1, 10, and 20 mg/mL). P <.0001, one-way ANOVA followed by Dunnett's multiple comparison test (n = 15). The adding of DMSO to the contracted rings without carvacol has no significant effect in suppressing the contractions as in the case of carvacol dissolved in DMSO [Figure 1b].

Effects of carvacrol on the vasocontractile responses evoked by potassium chloride in rat thoracic aorta

The contractile responses elicited by a single application of KCl (60 mM) were strongly inhibited in

a concentration-dependent manner in the presence of Carvacrol (1, 10, and 20 mg/mL). *P* <.0001, one-way ANOVA followed by Dunnett's multiple comparison test (n = 3) [Figure 2]. The relaxant effect was reversible and the spontaneous activity returned to normal after washing the tissue.

Effects of carvacrol on the vasocontractile responses evoked by U46619 in rat thoracic aorta

U46619, a Thromboxane A2 agonist (5-20 nm) produced a stable contraction (plateau) (mean = 0.51 ± 0.03 g, n = 7). Carvacrol (0.01, 0.1, 10, and 20 mg/mL) significantly inhibited the contractile responses evoked by U46619 (5-20 nm). *P* <.0001, one-way ANOVA followed by Dunnett's multiple comparison test (n = 4) [Figure 3].

Contractile responses to α,β-methylene ATP in the presence of U46619 in rat thoracic aorta

α,β-methylene ATP (1 μM), a P_2x receptor desensitizing agent, caused a transient contraction (mean = 0.21 ± 0.03 g, n = 7); these contractions were significantly enhanced in the presence of U46619 (**P <.01 vs. control Student's paired *t*-test [n = 7] [Figure 4]).

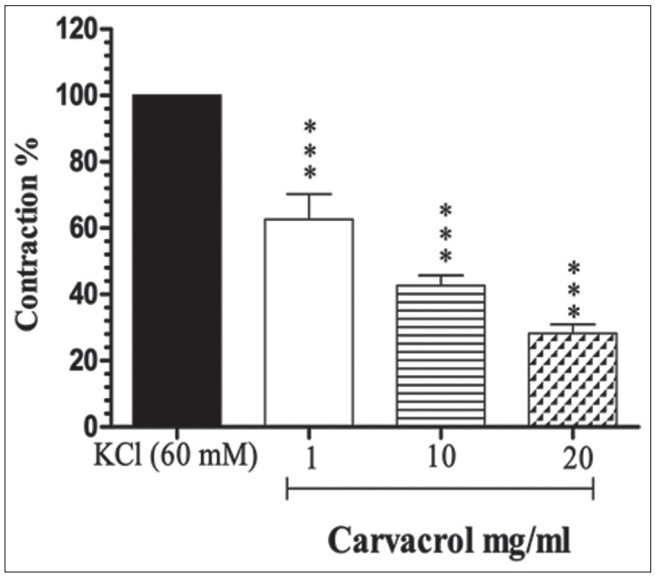

Figure 2: Effect of Carvacrol on the contractile responses in rat thoracic aortic precontracted with KCl (60 mM) (n = 3). Each bar represents the mean ± SEM. ***P <.0001 vs. control (one way ANOVA followed by Dunnett's multiple comparison test)

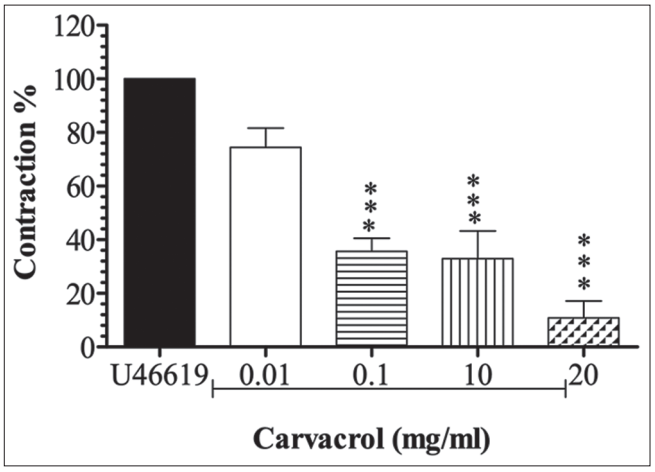

Figure 3: Effect of Carvacrol on responses to U46619 (5-20 nm) in rat aortic preparation (n = 4). Each bar represents the mean ± SEM. ***P <.0001 vs. control (one way ANOVA followed by Dunnett's multiple comparison test)

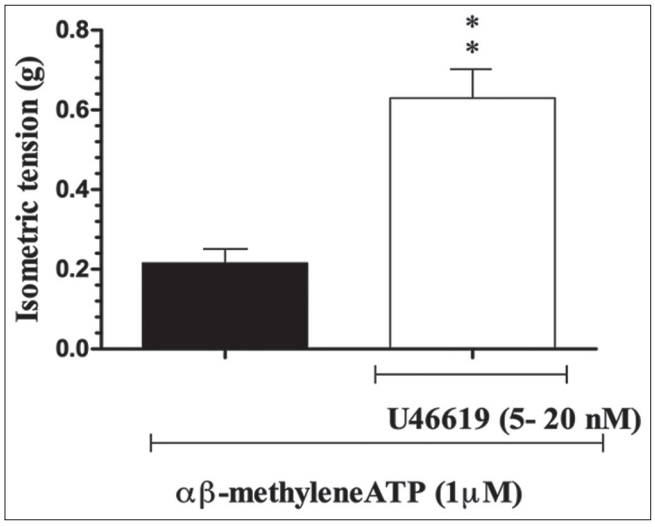

Figure 4: Responses to α,β-methylene ATP (1 μM) in the absence of U46619 (control) or in the presence of U46619 (5-20 nm) (n = 7) in rat aortic preparation. Each bar represents the mean ± SEM. **P <.01 vs. control (Student's paired *t*-test)

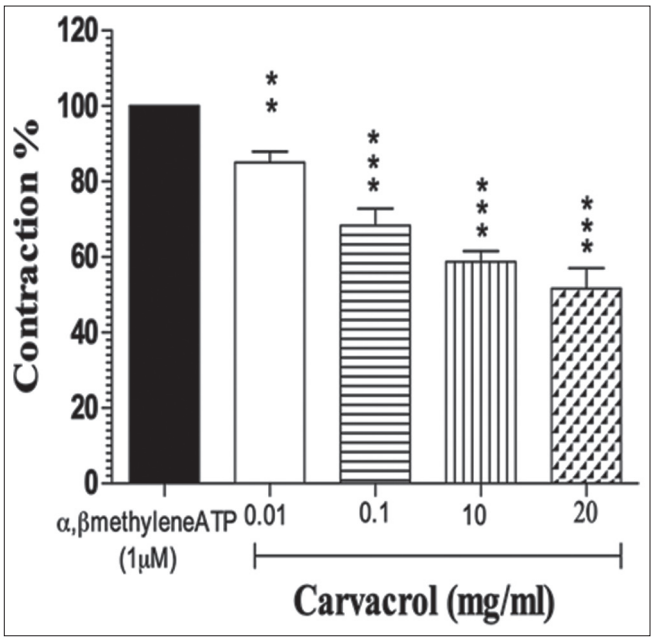

Figure 5: Effect of Carvacrol on contractile responses to α,β-methylene ATP (1 μM) in rat aortic preparation (n = 11). Each bar represents the mean ± SEM. **P <.001, ***P <.0001 vs. control (one-way ANOVA followed by Dunnett's multiple comparison test)

Effects of carvacrol on the contractile responses of α,β-methylene ATP in the presence of U46619 in rat thoracic aorta

Carvacrol (0.01, 0.1, 10, and 20 mg/mL) was significantly inhibited the contractile responses evoked by α,β-methylene ATP (1 μM) in the presence of U46619. *P* <.0001, one-way ANOVA followed by Dunnett's multiple comparison test (n = 11) [Figure 5].

Effects of carvacrol on the morphology of the rat thoracic aorta

Rats who received 15.6 mg/kg Carvacrol showed normal tunical appearance as the control animals. However, in rats who received 31.2 mg/kg Carvacrol, there was an increase in the thickness of tunica media as evident by the number of smooth muscle layers and laminae of elastic fibers. A further increase in the layers of smooth muscles and elastic fibers laminae was observed in the rats who received the highest dose of 46.8 mg/kg Carvacrol. It might be suggested that Carvacrol in high doses stimulates smooth muscles in the wall of aorta leading to an increase in the thickness of tunica media layer. Elastic fiber staining was performed to confirm that the increase in the thickness of tunica media in part was attributed to the increase of number of elastic fiber lamellae which was directly proportional to the concentration of Carvacrol.

Discussion

The present study has investigated the possible morphological and functional effects of Carvacrol on the smooth muscles of the thoracic aorta in the rat. The aortic rings were exposed to different stimulants separately [norepinephrine (NA) (1 μM), α,β-methylene ATP (1 μM), Thromboxane mimetic 9,11 – dideoxy -11 alpha, 9 alpha-epoxymethanoprostaglandin F2 alpha (U46619) (5-20 nm) and potassium chloride (KCl) (60 mM)] in the absence of Carvacrol and the presence of (0.01-20 mg/mL) Carvacrol.

It has been demonstrated that Carvacrol, the active ingredient of origanum, suppressed the contractile responses elicited by exogenous NA in a concentration dependent manner. It is known that NA evokes the noradrenergic component of the vascular smooth muscle contraction in the rat thoracic aorta.[14] α,β- methylene ATP has also been used, and for the first time, as an experimental tool to evaluate the purinergic component of the vascular tone. However, it has been reported that the purinergic component of the vascular tone was enhanced under any mechanism that increases vascular smooth muscle excitability such as raising the tone with U46619.[15] Therefore, in the present study, U46619 has been used to increase the vascular smooth muscle excitability of the rat thoracic aorta. In the presence of U46619, the purinergic component of the vascular smooth muscle contraction evoked by α,β- methylene ATP has increased in size and was also inhibited by Carvacrol in a concentration-dependent manner. Furthermore, Carvacrol has attenuated the contractile responses elicited by KCl in a concentration-dependent manner. In addition, Carvacrol has shown moderate dose-dependent changes in the ultrastructure of the rat aortic preparations studied by both LM and EM which is an increased number of pleomorphic mitochondria in the smooth muscles of the tunica media and by which confirms the high activity of smooth muscles fibers.

Using NA *in vitro* to examine the vascular smooth muscle activity serves as a model where sympathetic nervous system activation would be responsible for vasoconstriction since it is one of the sympathetic neurotransmitter triad produced by the sympathetic perivascular nerves. Data obtained in the present study have demonstrated that a single application of exogenous NA (1 μM) produced contractile responses. These responses were most likely mediated through α1-adrenoceptors; hence, it is well known that the noradrenergic component of the NA-mediated contraction usually occurs via α1- adrenoceptors.[16] In the present study, Carvacrol at different concentrations has significantly reduced the contractile responses induced by NA. These data are in consensus with a study showing that phenylephrine, an α1- adrenoceptors agonist-mediated vasoconstrictions were significantly inhibited by Carvacrol in rat isolated aorta.[17] In contrary to our results, phenylephrine-mediated vasoconstrictions in isolated rat aorta were not inhibited by Carvacrol (10^{-4} M).[14] This could be explained on the basis of the differences between the single concentration used in the mentioned study (10^{-4} M) and the present study in which a wide range of concentrations of Carvacrol were used (0.01, 0.05, 0.1, 0.5, 1, 10, and 20 mg/mL). Therefore, it is most likely that our data have shown strong evidence that Carvacrol has a clear interference with the signal transduction mechanisms induced by NA. Any agent which counteracts NA-mediated vasoconstriction is considered a vasorelaxent drug which exerts its effects by blocking noradrenergic receptors. In fact, many studies have reported that NA-induced contraction via α1- adrenoceptors is the complex result of the liberation of both intracellular and extracellular Ca^{+2} and the Ca^{+2} sensitization of the contractile apparatus.[18]

Data obtained in the present study have shown that α,β- methylene ATP evoked transient contractile responses in the rat thoracic aorta indicating the involvement of P_{2X} receptors in the mediation of these

contractile responses. Hence, it is well known that P_{2X1} receptor is considered to be the most important P_{2X} subtype reported in vascular smooth muscles.[19] Therefore, it is more likely that in this present study, α,β-methylene ATP–evoked contractile responses were mediated through P_{2X1} receptor. The involvement of P_{2X} receptors in the mediation of the purinergic response when activated by ATP or its analogue α,β-methylene ATP has been also reported in different studies. For instance, ATP has been the main neurotransmitter in rabbit hepatic, saphenous and jejunal arteries,[20] and in rat caudal artery.[21] Furthermore, ATP has been involved in the mediation of contractile responses in rabbit splenic artery,[22] rabbit mesenteric artery,[23] rat internal carotid, mesenteric, pulmonary, and thoracic aorta.[24]

In this study, the contractile responses evoked by α,β-methylene ATP were relatively small in size. This is expected, in fact, it is well known that the relative involvement of ATP as a sympathetic neurotransmitter in blood vessels is variable depending on different factors. For example, the level of vascular tone, in rat mesenteric arteries, raising intraluminal pressure has shown an increased role for ATP as a sympathetic neurotransmitter.[25]

Moreover, raising the vascular tone in the rat isolated perfused mesenteric arterial bed and revealed ATP as a sympathetic neurotransmitter.[26] A recent study has also revealed that ATP was more prominent as a functional sympathetic neurotransmitter when the porcine blood vessels were precontracted with U46619.[15] Therefore, in the present study, U46619 has been used to modify the excitability of the vascular smooth muscles in the rat thoracic aorta.

In the presence of U46619, the contractile responses produced by α,β- methylene ATP were significantly enhanced. These data are in line with a study showing enhanced contractile responses to α,β-methylene ATP in the porcine mesenteric arteries.[15] The mechanism by which U46619 enhances the nerve-mediated purinergic response is unknown; however, U46619 has been shown to produce vascular smooth muscle depolarization and to increase calcium levels.[27] Thus, creating a positive resting membrane potential under which the voltage gated calcium channels may get more probabilities to stay opened in response to P2X1 receptor stimulation. Another possibility is that U46619 may increase the production of second messengers activation or activation of Rho kinase and Ca^{+2} sensitization of contractile myofilaments.[28]

The enhanced contractile responses produced by α,β-methylene ATP in the presence of U46619 were significantly inhibited by Carvacrol. This indicates that Carvacrol may have influenced the mechanisms by which α,β-methylene ATP mediates its effects. It is most likely that α,β-methylene ATP mediated its effects through P_{2X1} receptors. P_{2X1} receptors are ATP-gated ion channels that allow primarily the entrance of Na^{+}, K^{+}, Ca^{+2}, and other small organic cation.[29] Therefore, it could be speculated that Carvacrol may have blocked the Ca^{+2} influx into the vascular smooth muscle membrane of the rat thoracic aorta. This assumption has been tested in the rat aorta where Carvacrol inhibited the contractile responses elicited by phenylephrine and it has been concluded that Carvacrol has altered Ca^{+2} influx.[18] Thus, it can be suggested that in the present study Carvacrol could have blocked the Ca^{+2} influx which is much needed for ATP or its analogue α,β-methylene ATP to mediate their effect through P_{2X} receptors.

In the present study, the KCl-induced contraction has been used as an experimental model to investigate the vascular tone in the rat thoracic aorta. It is well known that KCl causes depolarization of the muscle fibers which leads to the opening of L-type Ca^{+2} voltage-dependent channels and subsequent influx of extracellular Ca^{+2} which induces contraction.[18] Data obtained in the present study have demonstrated that KCl-mediated contractions in the rat thoracic aorta were also significantly inhibited in a concentration-dependent manner by Carvacrol. Similar data have been reported in the rat aorta where Carvacrol also inhibited the KCl-mediated contractions.[17] Furthermore, using the whole-cell configuration of patch clamp technique, Carvacrol suppressed L-type Ca^{+2} current in myocytes isolated from normal human and canine hearts.[30] Our data suggest that Carvacrol used in this study may have at least partially conveyed its effects in this manner.

To better understand the effects of Carvacrol on the morphology of the thoracic aorta, both light and electron microscopic studies were performed to observe the changes in the wall of the rat thoracic aorta. Rats who received 15.6 mg/kg Carvacrol showed normal tunical appearance as the control animals. However, in rats who received 31.2 mg/kg Carvacrol, there was an increase in the thickness of tunica media as evident by the number of smooth muscle layers and laminae of elastic fibers. A further increase in the layers of smooth muscles and elastic fibers laminae was observed in the rats who received the highest dose of 46.8 mg/Kg Carvacrol. It might be suggested that Carvacrol in high doses stimulates smooth muscles in the wall of aorta leading to an increase in the thickness of tunica media layer. Elastic fiber staining was performed to confirm that the increase in the thickness of tunica media in

part was attributed to the increase of number of elastic fiber lamellae which was directly proportional to the concentration of Carvacrol.

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Conflicts of interest

There are no conflicts of interest.

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