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THE ROLE OF K^+ AND CA^{2+} ION CHANNELS IN A-TERPINYLE ACETATE-INDUCED VASODILATION IN RAT'S AORTIC RINGS

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ABSTRACT:
The monoterpene, α-terpinyle acetate (TA) is a constituent of essential oils present in aromatic plants. Since the role of ion channels and endothelial hyperpolarizing factors in TA induced relaxation in rat's aorta is unknown, the current study aimed to study the mechanism underlying the vasodilatory effect of TA in isolated aortic rings. Terpinyle acetate induced a potent vasodilation in rat aortic rings with a percentage of relaxation of 63.79 %. The results of the role of K^+ channel subtypes in vasorelaxation revealed that both Kv and K_{ATP} played a major role since GLIB produced a maximum percent of inhibition in the relaxation produced by TA to ٨,٩١ %; this was followed by 4-AP in which the percent of inhibition reduced to ١٤,٩٥. On the other hand, K_{ir} played no role in the TA induced vasorelaxation since $BaCl_2$ did not produce any inhibition in aortic relaxation. Furthermore, also L-type Ca^{2+} channel played no role in TA induced relaxation since the L-type Ca^{2+} channel inhibitor Nifedipine did not reduce the percent of relaxation. Endothelium also played a considerable role in the induced vasorelaxation since, in denuded aorta, the percent of relaxation was reduced to 36%. Preincubation of the aortic ring with methylene blue, a soluble cGMP inhibitor also significantly reduced the TA induced relaxation to 16.39%. In contrast, preincubation with cyclooxygenase inhibitor Indomethacin did not produce any inhibitory effect on AT induced vasorelaxation. It can be concluded from these novel results that AT induced vasorelaxation involve the activation of K_V, K_{ATP} channels and at least partly dependent on endothelium via the activation NO-cGMP signal transduction pathway.

KEYWORDS: α-Terpinyle Acetate, K^+, L-type Ca^{2+} channels, vasodilation, aorta, rats.

1. INTRODUCTION

The plant's essential oils have been widely subjected to phytochemical studies (Abd El-Mageed *et al*., 2011). Monoterpenes represent an important active ingredients of the aromatic plant and may play a crucial role in many biological activities. The essential oils of *Eucalyptus tereticornis* induced myorelaxant effects on rat's isolated tracheal rings (Kheder, 2013). Monoterpenes, α- and β-pinene are involved in potentiating the action but are not responsible for its relaxant effects (Lima *et al*., 2010). Juca *et al*. (2011) found that the essential oils of *E. tereticornis* and its constituents decreased the gastric retention. In anesthetized rat's gastric strips, α- and β-pinene induced contraction, whereas enhanced the meal progression in the duodenum. On the other hand, the essential oil composition of *E. tereticornis* relaxed both the gastric strips *in vitro* but with enhanced relaxation in the duodenum (Juca *et al*. 2011). They suggested that the essential oils increased the gastric emptying, and its effect is partially due to its active constituent's α- and β-pinene. Lahlou *et al.* (2003) studied the effect of α-terpinen-4-ol on isolated aortic rings, precontracted with a depolarizing solution of K^+, α-terpinen-4-ol has induced vasorelaxation in a concentration-dependent manner. The hypotensive effect of monoterpene α-terpineol was first reported by Saito *et al*. (1996). In a study on α-terpineol-induced vasodilation in rat mesenteric vascular bed suggested that it involves NO pathway (Santos *et al*., 2011). Furthermore, α-terpineol-induced vasodilation in rat mesentric bed was inhibited completely by pretreatment with L-NAME, indicating the role of nitric oxide in the vasodilation (Magalhaes *et al*., 2008). On the other hand, Ribeiro *et al*. (2010) concluded that the vasodilation induced by α-terpineol was partially endothelium-dependent through producing nitric oxide and activating of the NO-cGMP pathway.

The appropriate modification in the structure of monoterpene, such as changes in the ethyl acetate group position and the presence of the aromatic ring in the *p*-methane skeleton may change the spasmolytic activity of monoterpene (Andrade *et al*., 2011). Thus, the current work is designed to investigate the role K^+ and Ca^{+2} ion channels and endothelium hyperpolarizing factors in TA induced vasodilation in rat's aorta.

2. MATERIALS AND METHODS

2.1 Tissue preparation

The animals were intraperitoneally injected with heparin (1500 units/ Kg body weight) followed by their anaesthetization with ketamin (40mg/kg) and xylazine (10mg/kg). The aorta was carefully isolated from the rat and transferred to aerate Kreb's solution with 95% O_2 and 5% CO_2. The isolated thoracic aorta was used in preparations with intact endothelium, where as in denuded preparations, the endothelium had been removed. The aortic rings (3-5 mm in length) were mounted between two stainless steel hooks, connected by a thread to a force transducer coupled to the trans bridge amplifier and PowerLab Data Acquisition system (ML 870, Power Lab, ADInstrument, Sydney, Australia), connected to a computer running chart software (Version 7). The isometric force produced was monitored and recorded (AL-Habib and Shekha, 2010). The experiments were performed in 10ml organ chambers filled with

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physiological kreb`s solution at 37ºC using the thermoregulating system with continuous water circulating throughout the double walled water jacket system and gassed with carbogen continuously (pH=7.4). The tension was set at 2g weight for 60 minutes and the solution was changed every 15 min. until a stable resting tone was obtained (Shekha, 2010).

2.2 Experimental protocols

In this study, cumulative dose-response relationships for the effects of α-terpinyle acetate (TA) at concentrations from $1*10^{-4}$- $3*10^{-3}$M were established for aortic rings. For all experiment, the vasorelaxant effects of TA acetate were studied in aortic rings precontracted with PE ($1X10^{-6}$ M), and were performed as follow:

2.2.1 Group I: The vasorelaxant effect of TA on aortic rings precontracted with phenylephrine (PE, 10^{-6}) was studied.

2.2.2 Group II: The role of K^+ channels in the development of vasorelaxation induced by TA was studied. The aortic rings were preincubated with K^+ channel blockers GLIB (10^{-5}), $BaCl_2$ (1mM) and 4-AP (1mM), for 20 min. prior to preconstruction by PE.

2.2.3 Group III: The role of Ca^{2+} channel in vasorelaxation induced by TA in aortic rings preincubated with L-type Ca^{2+} channel blocker nifedipine ($3x10^{-5}$) was studied for 10 min, prior to its precontraction by PE.

2.2.4 Group IV: The role of endothelial cells in vasorelaxation induced by TA was studied. The endothelium-denuded rings were firstly tested by the lack of any response to ACh (10^{-5}) followed contraction with PE to confirm the removal of endothelium.

2.2.5 Group V: The Role of Endogenous NO and NO-cGMP Pathway in TA-induced Vasodilation was studied. The aortic rings were preincubated with methylene blue ($1X10^{-5}$M) and Indomethacin ($3x10^{-5}$) for 10 min, prior to application of PE.

2.3 Statistical Analysis

The vasodilation response was calculated as a percentage of contraction produced by PE was expressed as the mean ± standard error of the mean (SEM). The base line tension was expressed as 0% relaxation, and the tension induced by PE defined as 100% relaxation. All data analysis were fitted with a Hill equation, which the mean effective concentration (Logs of IC_{50}) values were given as the geometric mean with 95% confidence intervals (95% CI), Using statistics program GraphPad Prism version 6.01. Two-way analysis of variance (ANOVA) was performed, supported with Bonferroni test when carrying out pair wise comparison between the same doses of different groups using GraphPad program. P-values less than 0.05 ($p< 0.05$) were considered significant. Symbols * mean $P< 0.05$, ** $P< 0.01$ and *** $P< 0.001$ for all graphs.

3. RESULTS AND DISCUSSION

3.1 Relaxant Effects TA on Aortic Rings

Dose-response curve for effect of TA against PE-induced contractions is shown in Figure 1. α- terpinyle acetate (TA) at concentrations from $1*10^{-4}$- $3*10^{-3}$M showed no relaxant effect in PE ($1X10^{-6}$) precontracted rat's aortic rings, whereas at concentrations of ($1*10^{-2}$ and $3*10^{-2}$), produced a significant ($P<0.05$ - 0.01) relaxant effect on rat's aorta with a relaxation of 63.79%. The Log IC_{50} and (Log IC_{50}'s of CI 95%) are shown in Table 1.

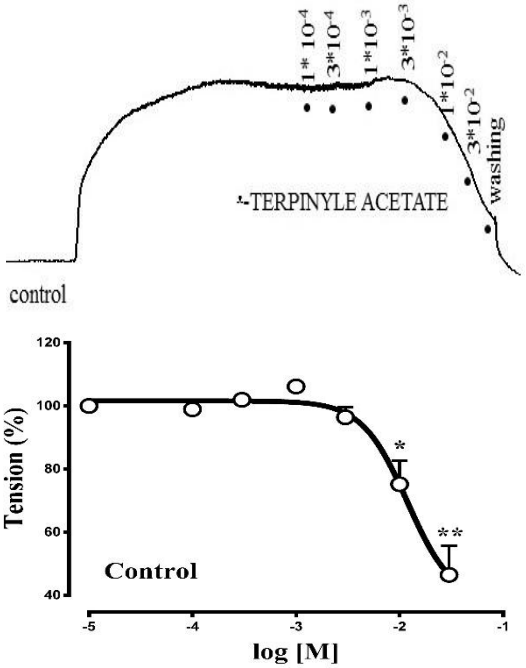

Figure 1. A typical chart view trace and a cumulative dose-response curve for the TA-induced vasodilation against PE ($1X10^{-6}$) in control.

The role of K^+ channel subtypes in TA-induced vasodilation was investigated using specific K channels blockers such as GLIB (10^{-5}), $BaCl_2$ (1mM) and 4-aminopyridine, (1mM), individually, 20 minutes prior to PE-induced precontraction of the aorta. Dose-response curves for TA-induced vasodilation against PE-induced contractions preincubated with K^+ channel blockers are shown in Figures (2 - 4). TA-induced relaxation was significantly ($P< 0.001$) reduced the effect of TA-induced vasodilation in preincubated aortic rings with K_{ATP} blocker (GLIB) which significantly affected TA induced relaxation at a concentration of ($3*10^{-2}$), Similarly, Kv channel blocker (4-AP) at TA doses ($1*10^{-2}$ to $3*10^{-2}$), in which the percent of relaxation was 14.95 %. In contrast, Kir channel inhibitor (BaCl2) at used concentrations did not affect TA induced relaxation in aortic rings except at the last dose with ($P< 0.05$), The Log IC_{50} and (IC_{50} of CI 95%) and percentage of relaxation calculated from TA dose-response curves are shown in Table 1.

These results revealed that both Kv and K_{ATP} played a major role in TA induced vasorelaxation since 4-AP and GLIB produced a maximum percent of inhibition in the relaxation produced by TA 14.95 %, and 8.91respectively. On the other hand, K_{ir} played no or less role in the process of vasorelaxation since $BaCl_2$ produce less inhibition on AT induced relaxation. It is not possible to compare these novel results since this represents a first study on the vasorelaxant effect of TA. However, it had been reported that 1, 8-cineol-induced vasodilation in isolated rat's aortic rings also involved both K_{ATP} and Kv channels, but with no role of Kca and Kir channels subtypes in this relaxation (Al-Habib *et al.*, 2013).

3.2 The Role of Calcium Channel in the TA- induced vasodilation

The cumulative addition of TA concentrations caused a concentration-dependent vasodilation in aortic rings preincubated with nifedipine. Dose-response curves for TA-induced vasodilation against PE-induced contractions in the presence and absence of Nifedipine are shown in (Figure 5). It is

clearly demonstrated that there is no significance difference between them, indicating that the L-type Ca^{2+} play no role in the TA induced relaxation of aortic ring. The percentage of relaxation, Log IC_{50} and (Log IC_{50}'s of CI 95%) are shown in Table 1. This indicates that L-type Ca^{2+} played no role in relaxant effect induced by TA. Also indicating that voltage-dependent Ca^{2+} channels did not involve in TA-induced vasodilation.

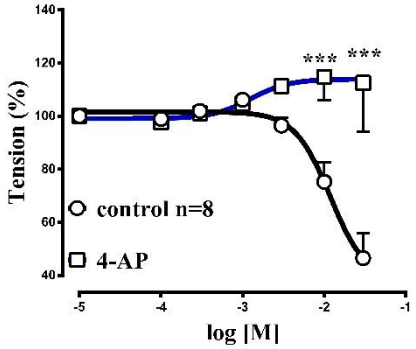

Figure 2. Cumulative dose-response curves for the TA-induced vasodilation in control and aortic rings preincubated with 4-AP (1mM), precontracted with PE (10^{-6}).

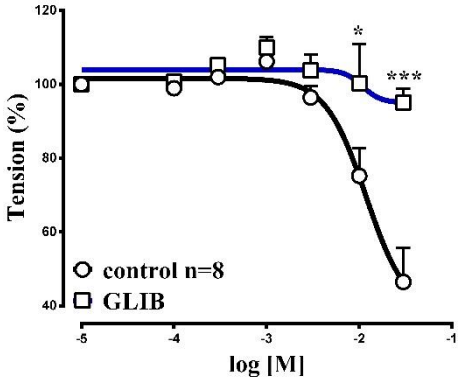

Figure 3. Cumulative dose-response curves for TA-induced vasodilation in control and aortic rings preincubated with GLIB (10^{-5}), precontracted with PE (10^{-6})

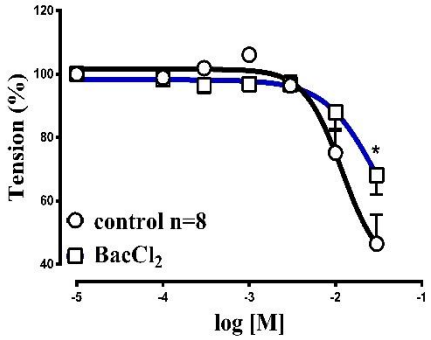

Figure 4. Cumulative dose-response curves for the TA-induced vasodilation in control and aortic rings preincubated with $BaCl_2$ (1mM), precontracted with PE (10^{-6}).

3.3 The Role of Endothelium in the TA- induced vasodilation

The dose-response curves for TA-induced vasodilation against PE-induced precontractions are shown in Figure 6. In the isolated aortic rings, the TA-induced relaxation in denuded aorta was slightly inhibited at concentrations ($1*10^{-2}$) and ($3*10^{-2}$) in which the inhibition was highly significance ($P<0.01$) only at the last dose used. Thus, the percentages of relaxation in both, endothelium-denuded and endothelium-intact preparation were more or less the same, except the highest dose (Table 1). removal of functional endothelium, significantly reduced TA-induced response, suggesting the vasodilation was endothelium-dependent. Furthermore, our result indicates that TA is an active monoterpene present in many plants, and induced vasodilation in rat aortic rings, at least partially via the endothelium-dependent release of NO. Ribeiro et al. (2010) demonstrated that α-terpinol induced vasorelaxation is mediated partially by endothelium via NO release and activation of the NO-cGMP pathway. terpineol is another active constituent present in *Eucalyptus camaldulensis* and able to induce a concentration-dependent vasorelaxation (Lahlou et al. 2003) at least partially by the endothelium mainly via NO release and activation of the NO-cGMP pathway

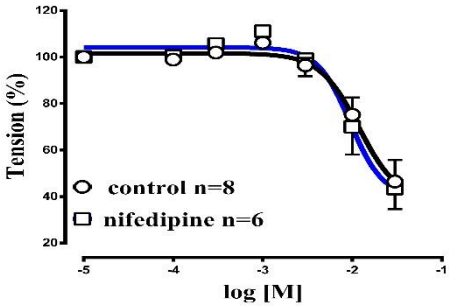

Figure 5. Cumulative dose-response curves for TA-induced vasodilation in control and aortic rings preincubated with Nifedipine ($3X10^{-5}$M), precontracted with PE (10^{-6}).

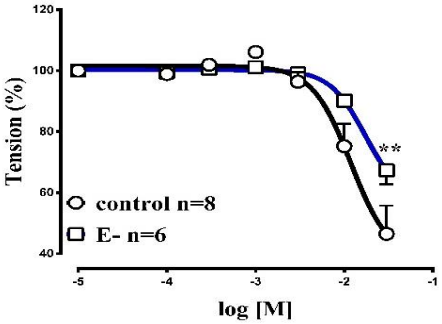

Figure 6. Cumulative dose-response curves for TA-induced vasodilation in endothelium-intact and denuded aortic rings precontracted with PE (10^{-6}M).

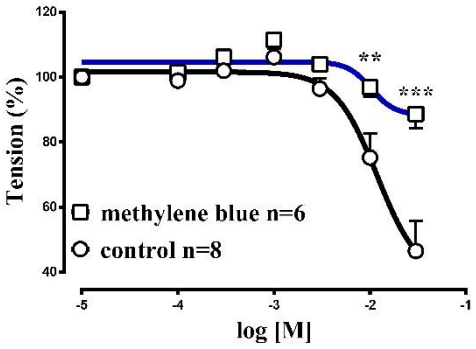

Figure 7. Cumulative dose-response curves for the TA-induced vasodilation in control & aortic rings preincubated with Methylene blue ($1X10^{-5}$M), precontracted with PE (10^{-6}M).

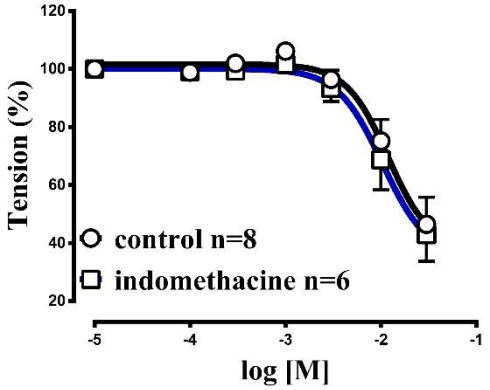

Figure 8. Cumulative dose-response curves for the TA-induced vasodilation in control and aortic rings preincubated with Indomethacin ($3X10^{-5}$M), precontracted with PE (10^{-6}M).

3.4 The Role of Endogenous NO and NO-cGMP Pathway in TA-induced Vasodilation

In both methylene blue and Indomethacin preparations, the cumulative addition of TA caused a vasodilation in a concentration-dependent manner. Dose-response curves for TA-induced vasodilation against PE-induced contractions in the presence and absence of methylene blue and Indomethacin are shown in Figures (7 and 8). The percent of relaxation was significantly ($P<0.001$) inhibited to (16.39%) in aortic rings preincubated with methylene blue at doses ($1*10^{-2}$ and $3*10^{-2}$), while in presence of indomethacin, the percent of relaxation remain almost unchanged. The percentage of relaxation, Log IC_{50} and (Log IC_{50}'s of CI 95%) for the relaxant response to TA are shown in Table 1.

The results of the effect of endothelium and cyclooxygenase pathway on TA induced vasodilation indicate that endothelium played a partial role in vasodilation induced by TA via the release of NO or activation of the NO-cGMP pathway which ultimately induces aortic relaxation. In studies on the effect of essential oils of *E. camaldulensis*, on rat's aorta and of *E. tereticornis* on the guinea-pig isolated aorta, indicated that its relaxant effect on isolated aorta and trachea may be due to the interaction between its monoterpenes constituents (Kheder, 2013;Coelho-de-Souza *et al.*, 2005). This indicates that vasodilation induced by the monoterpenes, α-terpineol is partially endothelium-dependent via the release of NO and activation of NO-cGMP- pathway (Ribeiro *et al.*, 2010). The TA-induced vasodilation was significantly reduced by the removal of endothelium, the guanylate cyclase inhibitor methylene blue but not by cyclooxygenase inhibitor indomethacin, suggesting a passive role of cyclooxygenase pathway in TA-induced vasodilation. The overall conclusion from the results on the mechanism of TA-induced vasodilation in rats aortic relays on the activation of K^+ channels subtypes namely, K_{ATP} and Kv channels and partially on endothelium via the release of NO and the activation of the NO-cGMP pathway which ultimately induce aortic vasodilation.

Table 1. The Log IC_{50} (Log IC_{50} of CI 95%) and percentage of relaxation for the effect TA-induced vasodilation on preincubated aortic rings with K^+ and Ca^{2+} channel blockers, methylene blue and Indomethacin, and denuded aortic rings

Essential oil								
Treatment	**Control**	**4-AP**	**GLIB**	**$BaCl_2$**	**Nifedipine**	**Denuded**	**Methylene blue**	**Indomethacin**
IC_{50}	0.012	0.001	0.011	0.016	0.01	0.015	0.01	0.01
95% CI IC_{50}	0.005035 to 0.027	0.0002 to 0.007	9.780e-007 to 120.6	0.003445 to 0.073	0.006 to 0.016	0.004349 to 0.054	0.004637 to 0.02273	0.005 to 0.023
Relaxation (%) ± SEM	63.79 ± 16.71	14.95 ± 1.42	8.91 ± 20.59	39.14 ± 19.15	64.58 ± 9.37	36 ± 15.94	16.39 ± 5.929	65.16 ± 13.995

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کورتیا لێکولینێ:

α-terpinyle acetate ئێکه ژ پێکهاتێن زەیتێت بێهنێ کو دیاربیت دناف روەکێت بێهنێ. دئەنجامدا هاتیه دیارکرن رولێ کەنالّێن ئایونا و هوکارێن خاڤبوونێ یێت اندوپیلیەمی ل سەر TA هەتا نوکه دیار نینه. لەوا ئارمانجا ئەڤ فەکولینه بو خاندنا جاوانیا فرەهبوونا ڕهیێن شادەمارێن سینگی ژ لایێ TA کو فرەهبوونەکا یان خاڤبونەکا بەرچاڤ ل سەر ڕهیێن شادەمارێن یێت دوور گرتین ب ریژەی ٦٣,٧٩% هەیە. و دیارە کر کو کەنالّێن K+ روله‌کێ کرنگ هەیە بو خاڤبوونا ڕهیێن شادەمارێن ژ بەر کو هەردوو کەنالێن K_V و K_{ATP}روله‌کێ مەزن هەبوو جنکی TA خاڤبوونەکا دیار هەبوو ل سەر ڕهیێن شادەمارێن ب ریژەی ٨,٩١% ئالوویرکرنا ب ریکرێ GLIB و هەروەسا 4-AP ریژا سەدی یا خاڤبوونێ١٤,٩٥%. ژ لایەکێ دی K_{ir} هیچ رول نەبوو بو خاڤبوونا ڕهیێن شادەمارێن بریکا TA جنکی بەرسڤدانا خاڤبوونێ ل ڕهیێن شادەمارێن ب ئالوویرکرنا بازنێن شادماران ب ریگرێن BaCl2 نەبوو. هەروەسا دیارکر کو رولێ کەنالّێن کالسیوم پشتی ئالوویرکرنا بازنێن شادمارا سینگی بریکا کەنالّێن کالسیوم ژ جورێن L ج رولێن خاڤبوونێ ل سەر TA دیار نەبوون جنکی ریژا خاڤبوونێ نه هاتیه خارێ. هەروەسا کارتێکرنا خاڤبوونێ بهێز ل سەر بۆریکێن خوینێ بێ اندوپیلیەم ل سەر کرژبوونا درستکری و ریژا سەدی یا خاڤبوونێ ٣٦%. بۆ هەلسەنگاندنا ڕۆلێ فاکتەرێن خاڤبووناداتاشراوکری ژ تفکلێن اندوپیلیەمی ڕیگرێن جیاواز هاتینه بکار ئینان، وەکی ئەندومیپاسین و methylene blue کێمبوونا بەرسڤدانا خاڤبوونێ ژ ئەگەرێن methylene blue ب ریژا ١٦,٣٩%. ژ لایەکی ڤه ج گوهرین ل سەر کارتێکرنا ئەندومیپاسین بو خاڤبوونا ڕهیێن شادەمارێن بریکا TA نەبوو. ئەم دشێن بێژین دئەنجامدا هاتیه دیارکرن کو خاڤبوون بریکا TA بەشداری دکەت د جالاکیا کەنالێن K_{ATP}و K_V وهەروەسا هیمەتەکا کێم ل سەر ژ تفکلێن اندوپیلیەمی ب ریکا NO- cGMP کو signal transduction pathway.

خلاصة البحث:

α-terpinyle acetate , أحد مكونات الزيوت العطرية الموجودة في النباتات العطرية.و بما ان دور القنوات الأيونية والعوامل الاسترخائية البطانية لـ TA غير معروفة لحد الان. لذلك فإن الدراسة الحالية تهدف إلى دراسة الآلية الكامنة وراء تأثير توسيع الاوعيه الدمويه من قبل TA لحلقات الشريان الأبهري المعزولة. وقد تبين ان TA سبب توسع ملحوظ للحلقات الشريان الابهر بنسبة الاسترخاء ٦٣,٧٩٪. وكشفت النتائج بان قنوات K^+ لها دور رئيسي في اسرتخاء الوعائي لان كلتا القنوات K_V وK_{ATP} لعبتا دورا كبيرا وقد تبين هذا من خلال الاسترخاء الذي نتج من قبل TA بالنسبة التثبيط ٨,٩١ % عند معاملتها ب GLIB وتبع هذا ب 4-AP فيه نسبة التثبيط خفضت إلى ١٤,٩٥. من جهة أخرى، K_{ir} لم يكن له اي دور في استرخاء الذي يسببه TA بسبب عدم وجود اي تثبيط في الاسترخاء للشريان الأبهر عند معاملتها ب $BaCl_2$. وبالاضافة قد تبين ايضا ان القناة الكالسيوم من نوع الـ L لم يكن لها اي دور للاسترخاء الناجم من قبل TAلان القناة الكالسيوم من نوع ال L لم تخفض النسبة المؤية للاسرخاء . أيضا كانت للبطانة دورا ملحوظا في ارتخاء الوعائي وتبين ذلك في الشريان المعري التي تم تخفيض الاسترخاء بالنسبة ٣٦%. التحضين المسبق للشريان الابهر ب الميثيلين الأزرق methylene blue الذي ايضا خفض الاسترخاء الناجم عن TA إلى ١٦,٣٩٪. وفي المقابل، التحضين ب الإندوميتاسين لم ينتج أي تأثير مثبط للاسترخاء الناجم عن TA. ويمكن أن نلخص من هذه النتائج الجديدة بان الاسترخاء الناجم عن TA تشارك في تفعيل القنوات K_V, K_{ATP} وعلى الأقل تعتمد جزئيا على البطانة عبر تفعيل NO-cGMP المسار التنبيهي الاشاري (signal transduction pathway)