Functional Effects of Alagebrium (ALT-711)–Isolated Rat Carotid Artery

İzole Sıçan Karotis Arter Preparatlarinda Alagebrium'un (Alt-711) Fonksiyonel Etkileri

Cigdem Toprak, Basar Sirmagul, Semra Yigitaslan

ABSTRACT

Objective: In our study, the effects of glycosylated protein cross-link breaker, alagebrium was investigated on isolated rat carotid artery using myography. Alagebrium showed vasodilator effect on carotid artery rings; particularly, this effect was significantly increased in endothelium-intact rings.

Materials and Methods: To clarify the vasodilator mechanism of alagebrium, different antagonists such as N(G)-Nitro-L-arginine methyl ester (L-NAME), glibenclamide, indomethacin, metoprolol, propranolol, tetraethylammonium, and calcium channel activator BAYK-8644 were used to reverse this effect.

Results: Relaxation% responses to alagebrium were more significantly increased in intact endothelium than in denuded arteries. Blocking vasodilation related to channels (K-ATP, PGI2, BKca) and receptors (ß1, ß2) did not reverse the relaxation response to alagebrium. Vasodilator response to alagebrium was only slightly decreased after L-NAME incubation and significantly decreased after BAYK-8644 incubation.

Conclusion: Results of present study suggest that the mechanism of alagebrium-induced vasodilator effect may include the blockage of L-type calcium channels and partially of the nitric oxide synthase enzyme.

Keywords: Alagebrium, calcium channel blockage, carotid artery, nitric oxide, vasodilatation

ÖZ

Amaç: Çalışmamızda, glikozile protein çapraz bağ kırıcısı olan alagebriumun sıçanlardan elde edilen karotis arter preparatları üzerindeki etkisi myograf yöntemi ile araştırılmıştır. Alagebrium karotis arter preparatlarında vazodilatör etki göstermiş ve bu etkinin özellikle endoteli sağlam damarlarda arttığı gözlenmiştir.

Gereç ve Yöntem: Alagebriumun vazodilatör mekanizmasını aydınlatmak amacıyla N(G)-Nitro-L-arginine methyl ester (L-NAME), glibenklamid, indometazin, metoprolol, propranolol, tetraetilamonyum gibi antagonistler ve kalsiyum kanal aktivatörü olan BAYK 8644 bu etkiyi geri çevirmek amacıyla kullanılmıştır.

Bulgular: Alagebrium % gevşeme yanıtları, endoteli sağlam damarlarda endoteli bozulmuş damarlara göre anlamlı derecede artmıştır.. Vazodilatasyon ile ilgili kanalların (KATP, PGI2, BKca) ve reseptörlerin (ß1,ß2) bloke edilmesi de alagebrium un gevşeme yanıtını geri çevirememiştir. Alagebriuma vazodilatör cevap L-NAME uygulanması ile hafif derecede azalırken BAYK-8644 uygulanması ile anlamlı derecede azalmıştır.

Sonuç: Çalışmamızın sonuçları; alagebriuma bağlı vazodilatör etki mekanizmasının L-tipi kalsiyum kanal blokajı ve kısmen de nitrik oksit sentaz enzim blokajına bağlı olduğunu göstermektedir.

Anahtar Kelimeler: Alagebrium, nitrik oksit, kalsiyum kanal blokajı, karotis arter, vazodilatasyon

Introduction

Advanced glycation end-products (AGEs) are harmful substances produced by non-enzymatic glycation of reducing sugars. Glycation is initiated by non-enzymatic reaction of the compounds having a carbonyl group with the amino group of proteins, nucleic acids, and lipids. Early glycation products, including reactive oxygen species (ROS), produced by oxidation reaction are transformed into AGEs by a number of irreversible reactions [1].

Overproduction and accumulation of AGEs having a key role in aging has been shown to result in several structural and functional disorders [2, 3]. Production of AGEs depends on the blood glucose concentration and the exposure time and may result directly from the hyperglycemia [4].

Advanced glycation end-products cross-link with tissue proteins, lipids, and DNA and result in a number of biochemical changes. Moreover, in patients with diabetes mellitus, they rapidly

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and increasingly accumulate in the tissue and organs, leading to diabetic complications including neuropathy, retinopathy, nephropathy, and atherosclerosis. Therefore, the inhibition of the production of AGEs may have beneficial effects on these complications [5, 6].

Alagebrium, also known as ALT-711, has enzymatic characteristics and breaks the covalent bonds formed in cross-linked proteins, allowing the protein to be detached and maintain its normal function. Even in the case of rebonding, alagebrium also breaks these bonds again [6].

Alagebrium has been developed for the treatment of cardiovascular disease such as heart failure and hypertension and shown to reduce the rigidity in major arteries, to increase the cardiac output, and to prevent the atherosclerosis in animal studies [7, 8].

On the other hand, in streptozotocin-induced diabetic mice, alagebrium leads to a decrease in AGE receptor (RAGE) expression, renal carboxyl-methyl lysine accumulation, and transforming growth factor-beta (TGF-β) release [9].

The aim in this study was to examine whether alagebrium, a cross-link breaker can induce an in vitro relaxation response with focusing on the underlying mechanisms.

Materials and Methods

This *in vitro* study used a total of 40 Sprague-Dawley male rats aging 3 months and weighing 250–300 grams for the experiments. Rats were killed for the experiments by cervical dislocation under light ether anesthesia. Study was approved by the ethics committee of our institution (number, 347-1, date 10.10.2013). Because of being an experimental animal study, no other informed consent was obtained.

Rat carotid artery myography

After incision of the skin and blunt dissection, right carotid artery was excised and put in ice-cold Krebs-Henseleit solution (composition in *g*/l: NaCl 6.9, KCl 0.35, $CaCl_2$ 0.29, $MgCl_2$ 0.24, KH_2PO_4 0.16, $NaHCO_3$ 2.1, and D-glucose 2). Fat and connective tissue was removed, and each arterial ring (3-4 mm in length) was mounted in a myography system (DMT 610 M, Danish Myo Technology, Denmark) in order to measure the isometric force. Rings were placed in chambers containing 10 ml of Krebs-Henseleit solution, which was heated to 37°C and gassed with 5% CO_2 and 95% O_2 mixture. A tension of 7 mN was applied to the arteries, and rings were allowed to equilibrate for 30 min. Tension was recorded on a chart recorder (MP150, Biopac System Inc; CA, USA).

For experiments using endothelium-intact arteries, integrity of the endothelium was verified by precontracting the arteries with 10^{-5} M phenylephrine (PE) (Sigma-Aldrich, St. Louis, MO, USA) and measuring the subsequent relaxation response to 10^{-6} to 10^{-3} M acetylcholine (Ach) (Sigma-Aldrich, St. Louis, Mo., USA). On the other hand, the luminal surface of some arterial rings was gently abraded by using a stainless-steel pin to remove the endothelium. Then, the removal of endothelium was verified by lack of a relaxation response to Ach. After assessment of the response to Ach, all rings were washed with Krebs–Henseleit solution and allowed to equilibrate for 30 min.

First, vasodilator response to cumulative doses of alagebrium (10^{-10} to 10^{-3} M) (Chemos, Germany) was verified in vessels precontracted with PE. Then, the vasodilator mechanism of alagebrium was evaluated by measuring the relaxation response to alagebrium in PE-precontracted arteries after 30-min incubation with 10^{-4} M L-NAME (NO synthase inhibitor) (Sigma-Aldrich, St. Louis, Mo., USA), 10^{-3} M metoprolol (beta-adrenergic receptor blocker) (Astra Zeneca, London, UK), 10^{-5} M indomethacin (COX enzyme inhibitor) (Deva Ltd, Istanbul, Turkey), 10^{-4} M tetraethylammmonium (calcium-dependent K-channel inhibitor) (Sigma-Aldrich, St. Louis, Mo., USA), 10^{-5} M glibenclamide (K-ATP channel inhibitor) (Sigma-Aldrich, St. Louis, Mo., USA), or 10^{-6} M BAYK-8644 (L-type calcium channel activator) (Sigma-Aldrich, St. Louis, Mo., USA). However, because vasodilator response to alagebrium was only slightly decreased after L-NAME incubation and significantly decreased after BAYK-8644 incubation, results for other agents were not presented here.

In order to verify the possible greater role of calcium channels in the relaxation response to alagebrium, 60 mM and 120 mM KCl were also used to precontract the vessels followed by administration of cumulative doses of alagebrium (10^{-10} to 10^{-3} M).

Percentage of contraction response (contraction%) and percentage of relaxation response (response%) were calculated as follows:

Contraction%*=[(maximum tension achieved by the contractile agent)−(basal tension)]×100/basal tension*

Relaxation%*=[(maximum tension achieved by the contractile agent)−(the minimum tension achieved by alagebrium)]×100/[(maximum tension achieved by the contractile agent)−(basal tension)]*

Data analysis and statistical test

Data analysis was employed with Minitab software version 16, (Minitab, Inc., Philadelphia, PA, USA) and Statistical Package for Social Sciences (SPSS) version 21 (IBM Corp.; Armonk, NY, USA). Normality of continuous variables was evaluated with Shapiro–Wilk test. Descriptive statistics of continuous variables were given as mean±SEM. Two-way ANOVA (one-factor repetition) was used for overall comparison, while pairwise comparisons were made with Tukey test. $p<0.05$ was considered as statistically significant.

Results

Responses of isolated rat carotid artery to alagebrium response to alagebrium in arteries precontracted with PE after incubation with L-NAME

Normal relaxation responses to alagebrium as well as relaxation responses to alagebrium after incubation with L-NAME were assessed in endothelium-intact (Et(+)) and endothelium-removed (Et(−)) arterial rings. There was a significant difference in relaxation% responses to alagebrium between Et(+) vessels and L-NAME–incubated Et(+) vessels ($p<0.05$) (Figure 1).

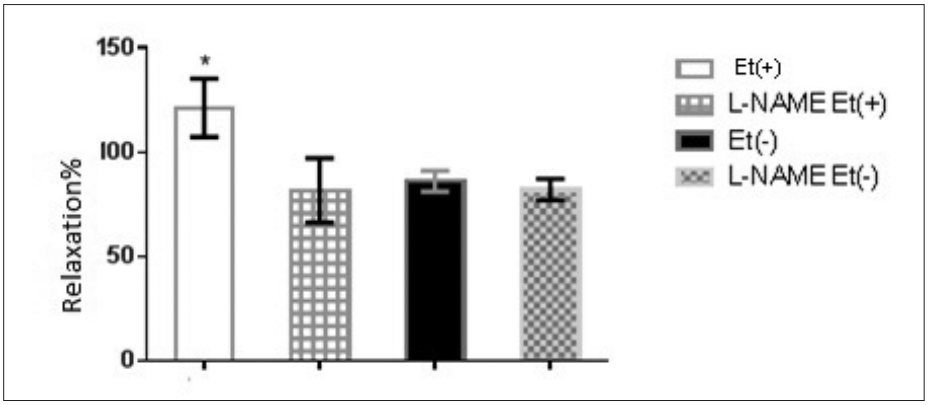


Figure 1. Normal relaxation responses to alagebrium as well as relaxation responses to alagebrium after incubation with L-NAME in endothelium-intact (Et(+)) and endothelium-removed (Et(−)) arterial rings. *$p<0.05$ compared to L-NAME Et(+)

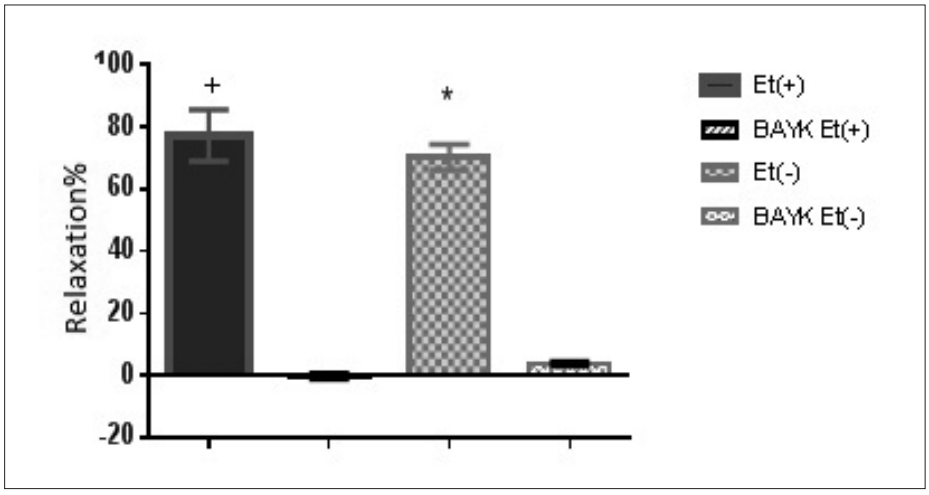


Figure 2. Relaxation% values to alagebrium in vessels precontracted with PE following BAYK-8644 incubation. +p<0.05 compared to BAYK-8644 ET(+); *p<0.05 compared to BAYK-8644 ET(−)

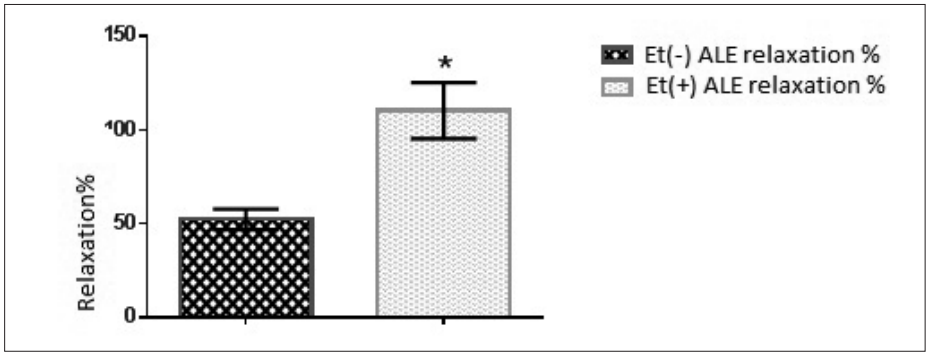


Figure 3. Relaxation% values to 10−3 M alagebrium in Et(+) and Et(−) vessels; *p<0.05 compared to ET(−) vessels

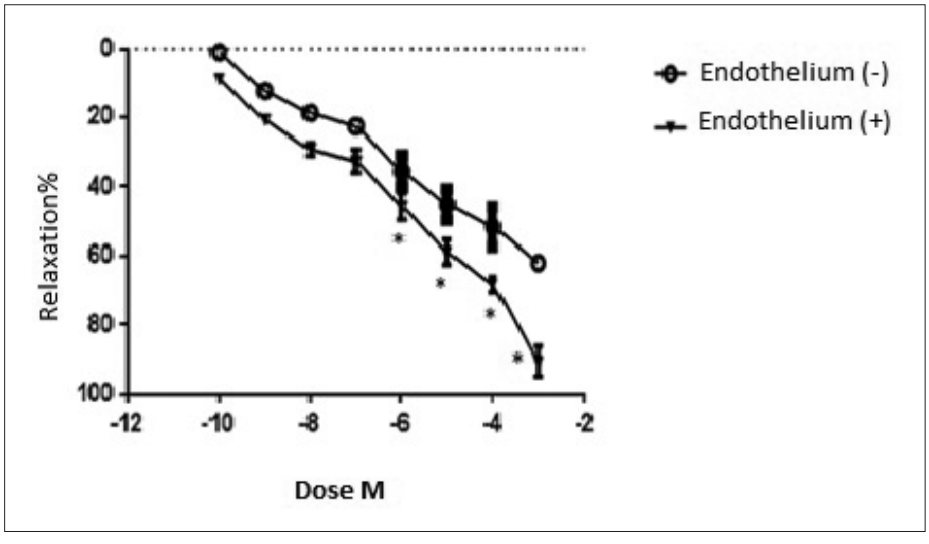


Figure 4. Relaxation% responses to cumulative doses of alagebrium in vessels precontracted with PE. *p<0.05 compared to ET(−) vessels

Response to alagebrium in arteries precontracted with PE after incubation with BAYK-8644

Normal Et(+) group showed lower relaxation% responses compared to those incubated with BAYK-8644 (p=0.0001). Et(−) arteries also showed significantly lower relaxation% responses after incubation with BAYK-8644 (p=0.0001) (Figure 2).

Response to 10^{-3} M alagebrium in Et(+) and Et(−) arterial rings precontracted with PE

Response of carotid artery preparations to alagebrium was assessed in both Et(+) and Et(−) rings after precontraction with PE with a significant difference between the groups in terms of relaxation% responses (p<0.05) (Figure 3).

Relaxation response to cumulative doses of alagebrium in Et(+) and Et(−) vessels precontracted with PE

Effect of cumulative doses of alagebrium (10^{-10} to 10^{-3} M) was assessed in carotid arteries precontracted with PE. There was a significant difference between Et(+) and Et(−) vessels in terms of relaxation% responses to cumulative alagebrium doses (p<0.05) (Figure 4).

Relaxation responses to cumulative doses of alagebrium in arteries precontracted with PE, 120 mM of KCl, and 60 mM of KCl

In the vessels precontracted with PE, there was no significant difference in relaxation responses between the alagebrium doses from 10^{-10} M to 10^{-7} M (p=1.0000). However, the doses of 10^{-6} to 10^{-3} M alagebrium resulted in significantly different relaxation responses compared to 120 and 60 mM KCl-contracted vessels with the dose of 10^{-3} M resulting in a significantly higher relaxation response than all other doses of alagebrium (p<0.01) (Figure 5).

In the vessels precontracted with 120 mM KCl, also no significant difference was present in relaxation responses between the alagebrium ^#^$doses from 10^{-10} to 10^{-4} M (p=1.0000), except the dose of 10^{-3} M alagebrium resulting in a significantly higher relaxation response than other doses of alagebrium (p<0.05) (Figure 5).

In the vessels precontracted with 60 mM KCl, also no significant difference was found in relaxation responses between the alagebrium doses from 10^{-10} M to 10^{-4} M (p=1.0000), except the dose of 10^{-3} M alagebrium resulting in a significantly higher relaxation response than other doses of alagebrium (p<0.05) (Figure 5).

Discussion

Natural aging process is associated with several metabolic changes including the production, accumulation, and binding of AGEs to their receptors which are the main factors damaging to the cells and tissues particularly in diabetic patients. Accordingly, glycation results in structural changes in the proteins and lipids, leading to the injury in several tissues mainly in the cardiovascular system. The cross-link products formed in the collagen and elastin result in a decrease in flexibility and elasticity of the heart and blood vessels.

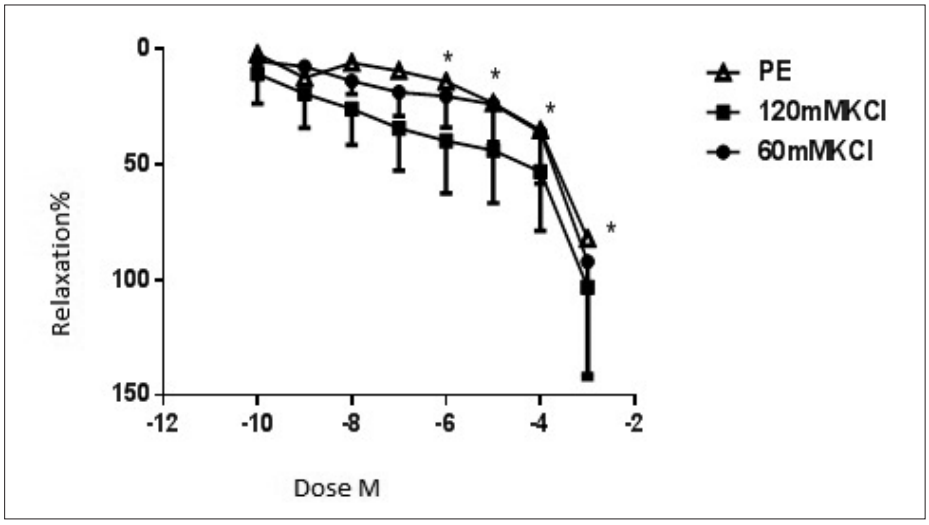


Figure 5. Relaxation% to cumulative doses of alagebrium in vessels precontracted with PE, 60, mM KCl and 120 mM KCl. *p<0.05 compared to 120 mM KCl group

It has been shown that AGE formation in the components of extracellular matrix (ECM) accelerates the formation of collagen cross-links in diabetes mellitus, contributing to the myocardial rigidity. Moreover, significantly increased AGE, AGE receptor (RAGE), and AGE receptor 3 (AGE R3) gene expression and increased connective tissue growth factor (CTGF) gene and protein expressions have been shown in the heart of diabetic patients. On the other hand, ALT-711 treatment has been found to decrease collagen solubility in the left ventricle (LV) and cardiac brain natriuretic peptide (BNP) and AGE levels as well as to eliminate the increase in AGE-R3, RAGE CTGF, and collagen III expression [9].

In a previous study of a carotid artery balloon injury in streptozotocin-induced diabetic rats, ALT-711 has been found to inhibit the proatherosclerotic effect of AGEs in the smooth muscle cells in rat aorta. Authors of that study suggested that the inhibition of neointimal proliferation by ALT-711 may be related to a decrease in extracellular matrix (ECM) products [10].

Recently, several agents preventing or decreasing AGE formation, breaking the AGE cross-links, or inhibiting the AGE-RAGE interaction are studied against the diabetic complications.

In the light of previous cardiovascular effects of ALT-711 in diabetic rats, we investigated the possible vasodilator effect of ALT-711 by studying the construction–relaxation response in isolated rat carotid artery preparations from healthy animals with using the responses to the agonists/antagonists in order to shed light on the possible underlying mechanisms.

We found significantly different relaxation response to ALE in Et(+) vessels compared to Et(−) vessels in carotid arteries precontracted with PE (Figure 1). When we compared the cumulative relaxation responses to ALE after agonist-mediated (by PE; 10^{-6} to 10^{-3} M) and hyperpolarization-mediated (by KCl; 60–120 mM), significantly higher relaxation response was found in arteries precontracted with 120 mM KCl compared to those precontracted with the same concentration of PE (Figure 5).

The significant relaxation response to ALT-711 found in both Et(+) and Et(−) vessels was significantly decreased after incubation with L-NAME with the decrease is being lower in Et(−) vessels, suggesting that an endothelial factor such as nitric oxide (NO) as well as possible other mechanisms may play a role in the relaxation response to ALT-711. Similar to our study showing that ALE contributes to the media layer-related non-endothelial relaxation responses, it has been shown in a previous study that AGEs mediate the thickening of intima-media layer which was reduced by ALT-711 breaking the glycosylated cross-links and preventing the accumulation of AGEs [11].

There are numerous human and animal studies reporting beneficial cardiovascular effects and possible underlying mechanisms with the use of ALT-711. In diabetic rats, collagen III was found to decrease, collagen solubility to increase, and RAGE and AGE-R3 mRNA levels to decrease when compared to the animals receiving vehicle after 4 weeks of treatment with ALT-711 [11, 12]. In vivo studies showed that ALT-711 improves LV function, decreases ventricular collagen, and decreases the rigidity of LV in elder diabetic rats with increasing the survival time [13, 14].

In humans, ALT-711 was found to improve the arterial compliance and decrease the pulse pressure in elder individuals [15]. In another study on 13 non-diabetic individuals with isolated systolic hypertension, arterial rigidity (carotid index) and brachial artery distensibility were evaluated by tonometer and Doppler echocardiography, and endothelial function was evaluated by flow-mediated dilatation (FMD). The authors have suggested that ALT-711 increases the peripheral endothelial function that was correlated with a decrease in vascular fibrosis and inflammatory markers. They have also suggested that AGE cross-link breakers can reduce cardiovascular risk by decreasing the arterial rigidity and vascular remodeling [15].

It is well-known that high extracellular KCl concentration activates Ca^{+2} entry to the voltage-gated calcium channels (VGCC) and particularly to the L-type calcium channel (LTCC), resulting in membrane depolarization and muscle contraction. BAYK-8644 is an LTCC activator increasing the intracellular Ca^{+2} concentration and inducing the contraction of vascular smooth muscle [16]. On the other hand, ventricular diastolic dysfunction is considered as the first step of the diabetic cardiomyopathy, and it is found in approximately 50% of the asymptomatic patients [17]. Because, intracellular Ca^{+2} homeostasis is a key factor for the connection between excitation and contraction and altered Ca^{+2} homeostasis in diabetes mellitus has been associated with the impairment in myocardial contractility and relaxation [18]. However, previous studies have failed to reveal the exact mechanisms underlying the impaired Ca^{+2} homeostasis as well as to define specific therapeutic targets for these patients [18, 19].

The main function of the sarcoplasmic reticulum (SR) is regulating the intracellular Ca^{+2} homeostasis and it is the major determinant of myocardial contraction and relaxation. The entry of Ca^{+2} into the cell through LTCCs results in activation of the Ca^{+2} release from SR. AGE cross-link breakers have been investigated to determine whether these agents can prevent the alterations in the SR-Ca^{+2} cycle, which lead to the cardiac dysfunction in diabetic patients [20]. The most important finding under this topic is that long-term ALT-711 treatment partially prevented the diastolic dysfunction in type-1 diabetic rats via partially improving the SR-Ca^{+2} move in the cardiac myocytes compared to the untreated diabetic rats [17, 18].

In the present study, incubation with BAYK-8644, a LTCC activator resulted in the complete elimination of the vasodilator response in both

Et(+) and Et(−) vessels, suggesting that the vasodilatation induced by ALT-711 may be related to the blockage of the calcium channels (Figure 2). Previous studies have suggested that abnormal Ca^{+2} release was found from the SR into the diabetic myocytes during the diastole and that ALT-711 stabilizes the ryanodine receptor (RyR)-mediated SR-Ca^{+2} release in the relaxation phase of the diabetic myocytes and thus maintains the Ca^{+2} homeostasis [19]. In a more recent study, ALT-711 has been reported to improve the antihypertensive effects of a calcium channel blocker, nifedipine in a rat model of diabetic hypertension, supporting results of the present study regarding the role of calcium channels in the vasodilator effects of ALT-711 [21].

The main limitation of this study is that we have indirectly showed the calcium channel blocking activity of alagebrium. However, if patch-clamp technique was used, the flow of calcium ions could be visualized. In addition, although we have tested the role of several mechanism with using the agents such as L-NAME (NO synthase inhibitor), metoprolol (beta-adrenergic receptor blocker), indomethacin (cyclooxygenase (COX) enzyme inhibitor), tetraethylammonium (calcium-dependent K-channel inhibitor), glibenclamide (K-ATP channel inhibitor,) and BAYK-8644 (L-type calcium channel activator), more mechanisms are involved in vasodilator and/or vasoconstrictor response such as Rho-kinase or many others.

In conclusion, although it has not been yet approved as a drug, the present study showed the *in vitro* vasodilator activity of ALT-711 in rat carotid artery preparations from healthy animals. Moreover, findings of this study suggest that this vasodilator effect may be related to the blockage of calcium channels as well as may be mediated by endothelial NO release. To best of our knowledge, this is the first study showing the functional consequences of the functional changes induced by ALT-711 treatment in the carotid artery of healthy animals. Future animal and human studies are needed to investigate both structural and functional benefits of ALT-711 in diabetes mellitus and hypertension.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Eskişehir Osmangazi University (Decision number: 347-1/Decision Date: 10.10.20139).

Informed Consent: Informed consent was not requested for this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - B.S.; Design - B.S.; Supervision - B.S, S.Y.; Resources - B.S.; Materials - B.S., Ç.T.; Data Collection and/or Processing - Ç.T.; Analysis and/or Interpretation - B.S., S.Y.; Literature Search - S.Y., Ç.T.; Writing Manuscript - S.Y., Ç.T., B.S.; Critical Review - B.S., S.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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