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Research Article
Enalapril and Valsartan Improved Enhanced CPA-induced Aortic Contractile Response in Type 2 Diabetic Rats by Reduction in TRPC4 Protein Level

[1]Sara Rowghani Haghighi Fard, [1]Masoumeh Emamghoreishi, [1]Ali Akbar Nekooeian and [2]Shirin Farjadian

[1]Department of Pharmacology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background and objective: Enalapril and valsartan have been used to treat vascular complications in diabetes. Considering the role of disturbed calcium hemostasis in vascular dysfunction in diabetes, the aim of this study was to evaluate the effects of enalapril and valsartan on vascular response in type 2 diabetic rats in relation to store-operated calcium entry and TRPC4 protein level and function. **Methodology:** Male Spargue-Dawley diabetic rats (n = 6 group^{-1}) were received distilled water, enalapril (20 mg kg^{-1} day^{-1}) or valsartan (20 mg kg^{-1} day^{-1}) for 6 weeks. Non-diabetic control rats were received distilled water for 6 weeks. Cyclopiazonic acid (CPA)-induced contractile response was measured in rat aortic rings in the presence and absence of ML204 (2.6 μM), a TRPC4 channel blocker. In addition, TRPC4 levels were measured in thoracic aorta using western immunoblotting method. **Results:** The CPA-induced contraction of vascular smooth muscle cell of aorta was significantly higher in diabetic control group as compared to non-diabetic rats. Enalapril and valsartan decreased aortic contractile response to CPA in diabetic rats to a level comparable to non-diabetic rats. The ML204 decreased aortic contraction in response to CPA in all studied groups in comparison to their relevant controls, although non-significant. The TRPC4 protein levels were significantly increased in aorta vascular smooth muscle cells of diabetic control group as compared to non-diabetic rats. Enalapril and valsartan significantly decreased TRPC4 protein levels in diabetic rats in comparison to diabetic control group. **Conclusion:** These findings suggest that enalapril and valsartan modify abnormal vascular response in diabetic rats through their effects on store-operated calcium entry which could be partially attributed to their effects on TRPC4 channels expression.

Key words: Diabetes, aorta, enalapri, valsartan, cyclopiazonic acid, Western blotting, ML204, TRPC4 ion channel

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Corresponding Author: Masoumeh Emamghoreishi, Department of Pharmacology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is associated with high frequency of mortality and morbidity due to its cardiovascular complications. Increased angiotensin II (Ang II) as a result of hyperglycemia[1-3] has been implicated in the pathophysiology of cardiovascular complications such as hypertension, restenosis and atherosclerosis in diabetes[4]. On the other hand, abnormalities in Vascular Smooth Muscle Cell (VSMC) have been related to atherosclerosis and its associated problems in diabetes[5,6]. The VSMC dysfunctions represented by impaired vasodilation or enhanced vasoconstriction[7-9] have been demonstrated in both chemical and genetic animal models of diabetes. Noteworthy, it has been shown that Ang II by increasing intracellular calcium influx in VSMC is a major cause of exacerbated response of VSMC to vasoconstrictors in diabetes[10,11]. Angiotensin Receptor Blockers (ARBs) and Angiotensin Converting Enzyme Inhibitors (ACEIs) widely used for prevention and treatment of cardiovascular complications in diabetes[12-17] have been shown to alleviate VSMC dysfunctions, however, their exact mechanism of action is not fully elucidated[18]. The Ang II play a role in VSMC dysfunction in diabetes by increasing intracellular calcium concentration. In addition phenotype changes of VSMC have been shown to have a shift in calcium channel expression with voltage-gated calcium channels down regulation and store-operated and receptor-operated calcium channel up regulation[19,20]. Therefore, it is possible that ACEIs and ARBs improve VSMC dysfunction through their effect on intracellular calcium homeostasis and expression of calcium channels.

The family of Transient Receptor Potential (TRP) channels has been introduced as an important modulator of intracellular Ca^{2+} [21,22]. Transient Receptor Potential Canonical (TRPC) channels are involved in regulating intracellular calcium homeostasis in VSMCs[23,24]. The TRPC channels may be activated either through activation of G-protein coupled receptors of the $G\alpha q$/11-PLCβ and receptor tyrosine kinases-PLCγ mechanisms known as receptor-operated calcium entry (ROCE) or by non-receptor mediated mechanisms such as inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) by cyclopiazonic acid known as store-operated calcium entry (SOCE)[24,25].

The most expressed TRPC isoforms in VSMC are TRPC1, TRPC4 and TRPC6, whereas TRPC3, TRPC5 and TRPC7 are less frequently expressed[26,27]. Among TRPC channels, TRPC4 is abundantly expressed in VSMC of aorta and its role in SOCE in VSMC has been shown[28]. Noteworthy, increased TRPC4 expression has been reported in Goto-Kaizaki animal model of type 2 diabetes and proposed to be a component of increased calcium influx exerted by Ang II Evans *et al.*[23]. In addition, changes in TRPC channels expression have been implicated in vascular dysfunction of diabetic human saphenous vein[29] and diabetic erectile dysfunction[30], abnormalities that have been attributed to increased calcium influx in diabetic VSMC[31,32].

Taken together, TRPC channels have been indicated to play a role in perturbation of intracellular calcium homeostasis by angiotensin II in diabetic VSMC dysfunction. The ARBs and ACEIs are indicated for improving cardiovascular complications of diabetes. Therefore, the aim of this study was to evaluate the effect of valsartan (an ARB blocker) and enalapril (an ACEI) on improvement of diabetic vascular dysfunction and its relation to SOCE and TRPC4 function/protein level in aorta rings of type 2 diabetic rats.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats (n = 24), weighting 200-250 g were obtained from Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences (Shiraz, Iran) and kept under standard conditions (12 h light/dark cycle, 22±2°C temperature with standard diet and water *ad libitum*) throughout the experimental period. Animals were acclimatized for 1 week before beginning of the experiment. All procedures were performed according to the guidelines for Care and Use of Animals of the University Committee.

Induction of type 2 diabetes: Rats (n = 18) were injected intraperitoneally (i.p.) with 110 mg kg^{-1} nicotinamide (NA) (Sigma-Aldrich Chemical Co., Steinheim, Germany) and 65 mg kg^{-1} streptozocine (STZ) (Teva Parenteral Medicine Inc., Irvine, CA, USA). Seven days later, blood glucose levels of rats were determined using a glucometer (Accu-check® active, Germany) and those with a fasting blood glucose between 126-200 mg dL^{-1} were considered as having type 2 diabetes[33,34]. One group of rats (n = 6) was i.p., injected distilled water to constitute the non-diabetic control group.

Starting from the day after the establishment of diabetes, rats were randomly divided into 3 groups (n = 6 $group^{-1}$) and received vehicle (distilled water) (DM-W), enalapril (20 mg kg^{-1}) (DM-E) or valsartan (20 mg kg^{-1}) (DM-Va) by oral gavage for 6 weeks. The non-diabetic control group was orally received distilled water for 6 weeks (ND-C).

At the end of 6 weeks, rats were anesthetized by i.p., injection of sodium thiopental (50 mg kg^{-1}). Then the thoracic aorta of each rat was dissected and cut into 6 equally pieces

(each 4-5 mm). Four pieces were immediately used for isolated tissue studies and the other 2 pieces were stored at -80°C for Western immunoblotting studies. Rats were allowed to bleed to death.

Isolated aortic ring studies: Isolated thoracic aorta was cleaned off blood. Adipose and connective tissues were removed from aortas by fine forceps and scissors and then aortas were cut into 4-5 mm rings. The endothelium was removed by gently rubbing the intima with rough surface wire[35]. The effectiveness of endothelium removal was functionally tested with the ability of 10^{-4} µM of acetylcholine to induce relaxation of aorta precontracted with 10^{-6} µM of phenylephrine. The de-endothelization was proven if the response to relaxation was less than 15%. The rings were mounted on hooks connected to force transducers in isolated tissue organ baths (K30, Hugo Sachs Electronik, Germany) filled with 20 mL physiological solution (pH = 7.4, temperature = 37°C) containing the following composition (mmol L^{-1}): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, D-glucose 11.1 and oxygenated with 95% O_2. Tension was recorded with a four-channel polygraph (model 705/1, Hugo Sachs Electronik, Germany). The rings were allowed to stabilize for 60 min at resting tension of 1 g with 3 washes every 20 min. Then the rings were challenged twice with 80 mM of KCl before the initiation of the given protocol. Then rings were equilibrated for another 60 min with 3 washes each 20 min. In each experiment, four rings from each rat's aorta were randomly treated with 10 µM cyclopiazonic acid (CPA) or DMSO or preincubated with 2.6 µM of ML204 (TRPC4 antagonist) or its vehicle (DMSO) for 45 min and then exposed to CPA. This dose of ML204 was chosen based on its IC_{50} from a previous study[36]. All compounds were added to each organ bath in a volume of 250 µL. The contractile response to CPA was determined as the percentage of the response to 80 mM of KCl.

Western immunoblotting study: The TRPC4 protein levels in rat thoracic aorta were determined by Western blotting as previously described with some modifications[37]. Thoracic aorta segments were homogenized in NP-40 buffer for 2 min at 4°C with a glass/teflon homogenizer (Eberbach Corporation, Ann Arbor, MI, USA). The lysate was centrifuged at 15000×g for 15 min at 4°C to remove insoluble debris. The supernatant was used for total protein quantification by Bradford method[38]. Each sample (20 µg total protein) was mixed with laemmli sample buffer (1:1), heated to 95°C for 4 min and loaded in duplicate into the polyacrylamide gel (SDS-PAGE) (4% stacking and 7.5% resolving gel). Twenty micrograms of common pool lysate was run in each gel to control between blot variations. The samples were electrophoresed at room temperature at 200 V for 40 min and then transferred to nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked with 5% skimmed milk for 2 h at room temperature followed by incubation with anti TRPC4 antibody (1:500) (Abcam, Cambridge, MA, USA) overnight at 4°C, then incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, Cambridge, MA, USA) to the primary antibody (1:5000) for 45 min. The lower portion of the membrane was blocked with 5% skimmed milk for 1 h at room temperature followed by incubation with anti β-actin antibody (Biolegend, San Diego, CA, USA) (1:10000) overnight at 4°C. The membrane was incubated with goat anti-rabbit HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA) to the primary antibody (1:5000) for 45 min. The primary and secondary antibodies were diluted in phosphate-buffered saline with tween 20. Enhanced chemiluminescence kit (ECL) (GE Healthcare) was used to visualize proteins on the membrane on x-ray film (Kodak, New York, NY, USA). The bands on the film were transformed into digital images using an HP scanjet G2710 scanner. Optical densities of TRPC4 bands were analyzed with ImagJv 1.47 and normalized against β-actin as internal control. The normalized optical densities (with arbitrary units) were used for statistical analysis. Between and within gel variations and the linearity of signal detection with loaded total protein were determined.

Statistical analysis: Data were presented as Mean±SEM. One-way analysis of variance (ANOVA) was used to compare mean differences between studied groups followed by Tukey's multiple comparisons tests. Student's t-test (parametric) and Mann-Whitney-U test (non-parametric) were used for comparison of mean differences between two groups. The SPSS version 18 was used for all statistical analysis and p-value<0.05 was considered statistically significant.

RESULTS

Effect of CPA on vascular contractility of thoracic aorta ring: In all studied groups i.e., ND-C, DM-W, DM-E and DM-Va groups, aortic contractile response to 10 µM CPA was significantly higher than that of DMSO ($p<0.05$) (Fig. 1). There were no significant differences in aortic responses to DMSO between four studied groups.

To compare aortic response to CPA between groups, contraction values of groups were normalized against their relevant controls (i.e., responses to DMSO) and analyzed by

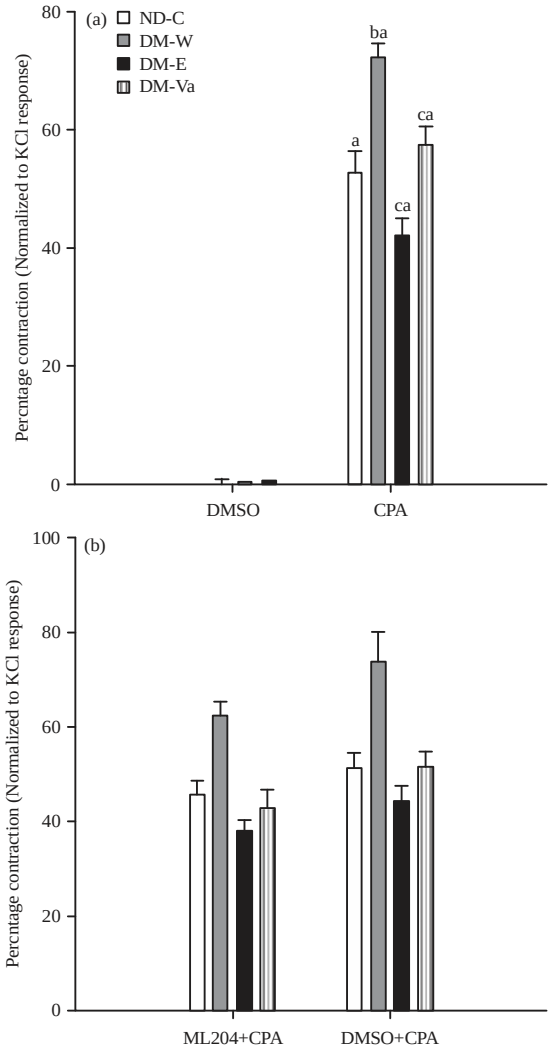

Fig. 1(a-b): Bar graphs represents Mean±SEM of percentage contraction of thoracic aorta rings to CPA, normalized to potassium chloride response in the (a) Absence and (b) Presence of ML204 for non-diabetic control group (ND-C), diabetic rats received vehicle (DM-W), enalapril (DM-E) or valsartan (DM-Va) (n = 6 group^{-1}), $^{a}p<0.05$ significant difference from DMSO treated rings, $^{b}p<0.05$ significant difference from ND-C group, $^{c}p<0.05$ significant difference from DM-W group

one-way ANOVA followed by Tukey's test. Contractile response of aorta to CPA was significantly higher in DM-W than that of ND-C ($p<0.05$) (Fig. 1). In addition, aortic contractile responses to CPA were significantly lower in DM-E (42±2.43%) and DM-Va (57±2.98%) groups in comparison to DM-W group (72.3±3.6%) ($p<0.05$) (Fig. 1). There were no significant differences in aortic contractile response to CPA between ND-C, DM-E and DM-Va groups (Fig. 1a).

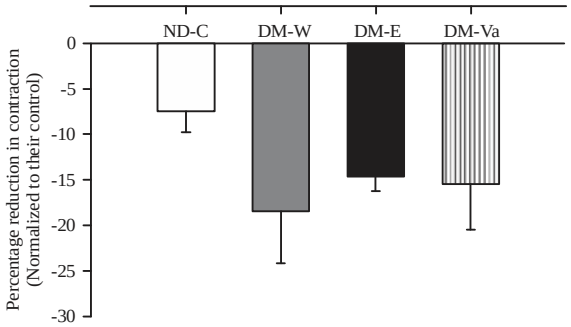

Fig. 2: Bar graphs represents Mean±SEM of percentage reduction in contractile response to CPA in the presence of ML204 from relevant DMSO-pre-incubated aortic rings for non-diabetic control group (ND-C), diabetic rats received vehicle (DM-W), enalapril (DM-E) or valsartan (DM-Va) (n = 6 group^{-1})

Effect of ML204 on aortic contractile response to CPA: Pre-incubation of aortic rings with ML204 reduced aortic contractile response to CPA in ND-C, DM-W, DM-E and DM-Va group in comparison to DMSO-pre-incubated aortic rings. However, these changes were not statistically significant (Fig. 1b). To compare the effect of ML204 on aortic response to CPA between groups, percentage changes from controls (DMSO) were calculated and analyzed by one-way ANOVA. There were no significant differences in reduced responses to CPA in the presence of ML204 between studied groups (Fig. 2).

Effects of valsartan and enalapril on TRPC4 protein levels: The immunoreactive bands of TRPC4 and β-actin were detected at the expected molecular masses of 112 and 42 kDa, respectively (Fig. 3). There were linear relationships between the signal intensities of β-actin ($r^2 = 0.98$) and TRPC4 ($r^2 = 0.98$) and total protein loaded in the range of 10-40 μg. The within-blot and between-blot coefficient of variance (CV%) were 4.9 and 6.7%, respectively.

The TRPC4 protein level was significantly higher in aortic smooth muscle of DM-W group as compared to ND-C group ($p<0.05$) (Fig. 3). Enalapril and valsartan significantly reduced TRPC4 protein levels in aortic smooth muscle of diabetic rats by 18 and 16%, respectively in comparison to DM-W control group ($p<0.05$) (Fig. 3). The TRPC4 protein levels were significantly lower in aortic smooth muscle of rats treated with enalapril (11%) and valsartan (8%) in comparison to ND-C group ($p<0.05$) (Fig. 3). There were no significant differences in aortic TRPC4 protein levels between valsartan and enalapril treated groups.

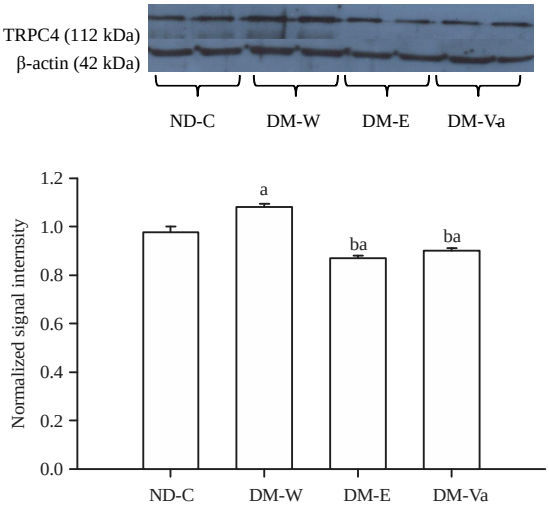

Fig. 3: Bar graphs represent the Mean±SEM of TRPC4 signal intensities (arbitrary units) normalized against β-actin as internal control and pooled lysates for non-diabetic control group (ND-C), diabetic rats received vehicle (DM-W), enalapril (DM-E) or valsartan (DM-Va) ($n = 6$ group^{-1}), $^{a}p<0.05$ significant difference from ND-C group, $^{b}p<0.05$ significant difference from DM-W group

DISCUSSION

To our knowledge, this study is the first to report that enalapril and valsartan restored increased contractile response to CPA and TRPC4 protein level in aortic smooth muscle of type 2 diabetic rats to a comparable response and levels of non-diabetic aorta. These findings suggest that enalapril and valsartan improve vascular dysfunction in diabetes through their effects on SOCE and TRPC4 protein level.

Increase in cytosolic calcium concentration by calcium influx through plasmalemmal calcium channels plays a critical role in vascular smooth muscle cell contraction. The CPA by activating store-operated calcium channels triggers capacitative calcium entry which can causes vasoconstriction in smooth muscle cells[39].

This study showed an increased contractile response to CPA in aortic smooth muscle of type 2 diabetic rats in comparison to non diabetic rats. This is in consistent with a previous study that showed exaggerated CPA induced SOCE and contraction of the saphenous vein from patients with type 2 diabetes as compared to the vessels from subjects without diabetes[29]. In addition, increased CPA-induced contraction of the longitudinal smooth muscle of rat urinary bladder has been shown in diabetic rats in comparison to the non-diabetic control rats[40]. On the other hand, decreased CPA-induced SOCE and contraction of endothelium denuded caudal artery strips has been reported in Goto-Kakizaki genetic model of type 2 diabetes mellitus[41], which this discrepency may be due to the use of genetic animal model and stages of diabetes and tissues in these different studies.

The CPA is a SERCA inhibitor that triggers capacitative calcium entry via the opening of store operated calcium channels that depolarizes the plasma membrane followed by opening voltage gated calcium channels and allowing more calcium entry into the cell. This study showed an increase CPA-induced aortic contraction in diabetic rats which suggests that store-operated calcium entry may be disturbed in aortic vascular muscle cells of diabetic rats. This is in agreement with previous studies that indicated abnormal calcium homeostasis[42], altered intracellular level and distribution of calcium[43] and increased capacitative calcium entry[44] in diabetic vascular smooth muscle cell.

In this study, treatment of diabetic rats with enalapril and valsartan decreased aortic contraction in response to CPA to a comparable level of non-diabetic rats. Until now, there are no other reports evaluating the effects of enalapril and valsartan on capacitative calcium entry in aorta of diabetic rats to compare with the current results. However, in line with this finding, it has been shown that administration of enalapril could decrease vascular responsiveness to vasoconstrictors, such as phenylephrine and produce increased relaxation response to acetylcholine in diabetic rats[45]. The results of the present study suggest that enalapril and valsartan modify store-operated (SOCE) calcium entry in vascular smooth muscle cells of diabetes which in turn, may be the mechanism by which these drugs have beneficial effects in diabetic vasculopathy as indicated in clinic.

To further elucidate the mechanism of action of valsartan and enalapril on SOCE, the effect of these drugs on TRPC4 function and protein level were evaluated. In the present study, administration of TRPC4 blocker (i.e., ML204) has not completely reversed the increased CPA-induced contraction in aortic VSMC of all studied groups in comparison to their respective controls. Our expectation was to see significant reduction in diabetic control group by the use of ML204 if TRPC4 was partly involved in SOCE in increased response to CPA. Since, we did not see significant difference in reduction of contraction by CPA in diabetic control, it is most speculated that the dose of ML204 was not sufficient to reverse the activity of TRPC4 involved in this contraction or the high variability of the data was concerned. Since, no significant difference in the action of ML204 between studies group was seen, we could not suspect the effects of enalapril and valsartan on TRPC4 function. Certainly, further studies using

different concentration of ML204 or higher number of rats are needed to address this issue. However, the observation of partial reversal of contractile response to CPA in the presence of ML204 suggests that TRPC4 channels might be, at least partly, involved in disturbed capacitative calcium entry in VSMC of diabetic rats. So, for the better underastanding of the effects of enalapril and valsaratn on TRPC4, Western blotting study was carried out.

Higher TRPC4 levels in aorta of type 2 diabetic rats were observed in the present study. This is in agreement with a previous report of increased TRPC4 protein levels in aortic smooth muscle in Goto-Kakizaki diabetic rats[23]. Moreover, increased TRPC4 protein level has been shown in corpus smooth muscle of STZ induced diabetic rats[30]. In addition, increased mRNA, but not protein level of TRPC4 has been reported in saphenous veins of type 2 diabetic patients[29] that all were contributed to abnormal increase SOCE. Findings of this study suggest that increased TRPC4 protein level may partly play a role in disturbed SOCE and increased contractile response to CPA in aortic VSMC of diabetic rats. Interestingly, in the present study it was revealed that enalapril and valsartan reversed the up-regulation of TRPC4 protein level in aortic diabetic type 2 rats. This observation further supports the involvement of TRPC4 in contractile response of aorta of diabetic rats which can be modified by enalapril and valsartan. This is the first study that confirms the effects of enalapril and valsartan on TRPC4 protein levels in diabetes. However, it has been shown that ramipril and valsartan could reduce expression of TRPC3 protein in aortic smooth muscle cells of systemic arterial hypertension[46]. Taken together these observations suggest that ACEIs and ARBs might affect vascular functions by altering the expression of TRPC channels.

In the present study, enalapril and valsartan modified SOCE and TRPC4 protein level in VSMC of diabetic rats. The potential mechanism by which enalapril and valsartan exert these effects may be through their action on Ang II. There are evidences that ACEIs and ARBs inhibit deleterious effects of Ang II on vascular smooth muscle cells in diabetes[11,47]. Enalapril decreases Ang II production and valsartan antagonize Ang II function through binding to its receptor i.e., AT1R. Following binding to its Gq-coupled receptor, Ang II increases intracellular calcium concentration through voltage-gated calcium channels (VGCC) and voltage independent calcium channels. However, previous studies have indicated that a major component of calcium influx caused by Ang II is through store-operated calcium channels, such as TRP channels. Noteworthy, it has been demonstrated that Ang II-induced Ca^{2+} influx was mediated by 1/4/5 subgroup of TRPC channel in diabetic aortic smooth muscle cells[12,48,49]. Therefore, it is possible that valsartan and enalapril modulate capacitative calcium entry in aortic vascular muscle cells of diabetic rats by inhibiting the effects of AngII on SOCE and TRPC channels.

Changes in TRPC4 protein level might be through altering gene expression or protein stability and/or increasing protein degradation. There are evidences that TRPC4 gene expression have been changed in pathological conditions through different transcription factors[25] such as AP-1 and cyclic AMP response element-binding protein (CREB)[50]. Furthermore, losartan[51,52] has been shown to inhibit CREB activation which was dependent on Ang II action. Therefore, it is likely that enalapril and valsartan affected TRPC4 channel expression by CREB activation dependent of their effects on Ang II. However, this notion has to be addressed in future studies.

Overproduction of ROS has been described as a pathological process in diabetes through different mechanism that Ang II is one important suspect for ROS induction[53,54]. There has been shown that increase of ROS in diabetes changes TRPC mRNA or protein expression[55,56]. The ROS in different cell types and conditions could use diverse pathways, which can cause the upregulation or downregulation of a particular TRPC isoform. Moreover, there have been evidences that confirm the role of ROS in the development of diabetic complications[57,58]. Thus, oxidative stress may be a general mechanism for diabetes-associated TRPC protein dysregulation. As antioxidant properties of enalapril and valsartan[45,59,60] has been shown in different studies, it is possible that in this study these drugs by inhibiting the action of Ang II in producing ROS might have regulated TRPC4 protein level in diabetic state.

From different point of view, enalapril and valsartan may act on SOCE and TRPC4 protein levels by mechanisms other than their effects on Ang II. First possibility is through direct effects on Ca^{2+} handling in aortic smooth muscle cells as previously shown for enalapril and captopril[45,61]. Second, the effects of enalapril and valsartan on TRPC4 channel gene expression might be through transcription factors independent of the action on Ang II[25]. In this regard, it has been reported that TCV-116, an ARB, inhibited the action of transcription factor AP-1 which was independent of its action on Ang II[62].

This study had some limitations. We did not examine other TRPC channel expression and function in order to clarify the effect of enalapril and valsartan on other TRPC channels since most of TRPC channels works as heterotetramers for activation and also the contribution of other TRPC channel in vascular dysfunction in diabetes should be evaluated. Also, it is better to examine the expression of other component of

SOCE, such as two proteins called STIM1 and Orai1 that their changes have been reported in diabetes[63]. In addition, the mechanism of increased expression of TRPC4 in the aortic smooth muscle of diabetic rats remains unknown. Further study is needed in regard to this issue.

CONCLUSION

In summary, this study is the first that demonstrated increased aortic contractile response to CPA that is accompanies with increased TRPC4 protein level in STZ-induced type 2 diabetic rats that have been reversed by enalapril and valsartan. These findings suggest that enalapril and valsartan may modify abnormal vascular response and capacitative calcium entry in diabetic rats, at least partly, through their effects on TRPC4 channels. This study, provide a new prospective on the pathogenesis of diabetic vascular disease through changes in TRPC4 channel. Moreover, our data open a new avenue the mechanism of action of ACEIs and ARBs in treating vascular disease in diabetes which leads to novel drug development in the fields of cardiovascular complications in diabetes.

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