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Role of Endothelium on Cyclopiazonic Acid-induced Vascular Contractions in Rat Aorta

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Introduction

Spacio-temporal changes of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are known to play central role in numerous cellular processes such as muscle contraction, gene expression, development, proliferation and apoptosis[1]. While global increases in $[Ca^{2+}]_i$ in vascular smooth muscle cells (VSMCs) elicit contraction[2, 3], $[Ca^{2+}]_i$ elevation in endothelial cells (ECs) causes vasorelaxation by triggering synthesis and/or release of vasoactive substances including nitric oxide (NO) or prostanoids[4, 5]. Physiological or pathological stimuli result in elevation of $[Ca^{2+}]_i$ via voltage-operated Ca^{2+} channels (VOCCs), receptor-operated Ca^{2+} channels (ROCCs) as well as store-operated Ca^{2+} channels (SOCCs)[2, 6, 7]. Store-operated Ca^{2+} entry (SOCE) is activated by depletion of intracellular Ca^{2+} stores via inositol 1,4,5-triphosphate (IP_3) as well as other Ca^{2+}-releasing signals. Ca^{2+} stores can also be depleted by uncompensated Ca^{2+} leakage from sarcoplasmic reticulum (SR) that is caused by selective sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitors, cyclopiazonic acid (CPA) and thapsigargin [8, 9], both of which are known to induce SOCE[10].

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Store-operated Ca^{2+} entry, also called capacitative Ca^{2+} entry[11, 12], is critically important mechanism for the cell in terms of retaining $[Ca^{2+}]_i$ elevation and refilling intracellular Ca^{2+} stores despite limited ligand-receptor interaction[13-15]. Previously, it has been suggested that SOCE is tightly restricted within non-contractile subsarcolemmal compartments enclosed by superficial SRs and is coupled to vascular contraction via activation of co-localized protein kinase C (PKC) in the same region[3, 16]. In addition to mechanisms in VSMCs, functional importance of endothelial secretions in regulation of vascular tone has been first described in 1980[17]. Endothelium-derived vasoconstricting and relaxing substances are suggested to be controlled by spacio-temporal changes of $[Ca^{2+}]_i$[18]. SERCA blockade induces vasodilatation in all intact vascular tissues via elevating endothelial $[Ca^{2+}]_i$.[19]. This $[Ca^{2+}]_i$ that is raised in ECs triggers vasorelaxation through endothelial nitric oxide synthase activation (eNOS) followed by NO release[20, 21]. eNOS has been shown to be localized near specialized flask-shaped pits of 60-80 nm diameter on the cell membrane. Moreover, eNOS has been suggested to be inactivated by a caveola-specific protein, caveolin-1 (Cav-1), in these specialized sub-sarcolemmal regions[22, 23].

In an earlier report, transient contractions induced by SERCA inhibition have been suggested to be due to thromboxane A_2 (TxA_2) that is released from endothelium in isolated mouse aorta[24]. On the basis of several conflicting observations, we questioned the mechanism of persistent vasoconstriction in the case of endothelial dysfunction that accompanies SERCA downregulation in the rat aorta.

Material and Methods

All animals received care according to the criteria outlined in the "Guide for Care and Use of Laboratory Animals" prepared by the National Academy of Science, also adopted by the Animal Ethics Committee (Ege University, Faculty of Pharmacy, B.30.2.EGE.0.03.00.01/47). Rats (Sprague Dawley, male, 300–350 g) were asphyxiated with CO_2 and the thoracic aorta was isolated, immediately placed in physiological salt solution (PSS: Krebs-Ringer bicarbonate solution containing (mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.17 $MgSO_4$, 1.2 KH_2PO_4, 11.1 Glucose and 25 $NaHCO_3$) and cleaned of

surrounding fatty tissue[25]. Each aorta was cut into 3 mm-long circular segments. In some experiments, the endothelium was removed by gently rubbing the intimal surface with stainless-steel wire. Ring preparations were mounted between two stainless-steel hooks in organ baths each containing 25 ml PSS (37°C) gassed with 95% O_2 and 5% CO_2. The upper wire was connected to an isometric force-displacement transducer (FDT 10-A Commat, Turkey) which is mounted on a displacement unit allowing a fine adjustment of tension. The preparations were allowed to equilibrate for 45 min in PSS. During this period the organ baths were washed with fresh (37°C) PSS solution every 15 min. After 45 min, each ring was gradually stretched in 0.5 g increments to the previously established optimal point of the resting tension for rat aortas, 20 mN. Once at their optimal length, the segments were allowed to equilibrate for 30 min before experimentation. Following stabilization period, tissues were challenged twice with phenylephrine (PE) (1 μM for intact, 300 nM for endothelium-denuded vessels). Functional presence of endothelium was confirmed by acetylcholine (ACh)-induced relaxations (10^{-9}-10^{-4} M) in PE-precontracted tissues. Experimental protocol for intact (+E) and endothelium-denuded (-E) aortic segments: a) control, b) 30 min 200 μM NOS inhibitor, N^{ω}-nitro-L- arginine methyl ester (L-NAME) and c) 30 min L-NAME + 10 μM cyclooxygenase (COX) inhibitor indomethacin (IND) (to eliminate endogenous prostanoid synthesis) preincubation prior to administration of CPA at concentrations (10 μM) that deplete agonist-sensitive SR Ca^{2+} stores[8].

All organ bath measurements were recorded using a digital data acquisition system (Biopac System Inc., USA). Force was normalized to cross-sectional area [Force/Area= (change in force x circumference)/ 2 x wet weight].

Drugs

PE, ACh, IND, L-NAME and CPA were from Sigma-Aldrich Chemical Co. Stock solutions and subsequent dilutions of PE, ACh and L-NAME were prepared in distilled water. Indomethacin and CPA were dissolved in ethanol and DMSO, respectively. Further dilutions of CPA and IND were made in distilled water. Final bath concentration of

DMSO and ethanol did not exceed 0.1% to eliminate the vehicle's vasorelaxant effects[26].

Statistics

All values are expressed as mean±S.E.M. "n" represents the number of animals used. Statistical significance was evaluated using Newman Keul's test for multiple comparisons. $P<0.05$ was considered significant.

Results

The effects of CPA on endothelium-derived relaxations were tested in a group of intact vessels. Cyclopiazonic acid induced concentration-dependent relaxations in PE-precontracted tissues incubated with indomethacin (Figure 1). However, CPA at concentrations above 10 µM elicited contractions (Figure 1). CPA concentration was not increased above 30 µM due to the limitation of DMSO as described in the Materials and Methods section.

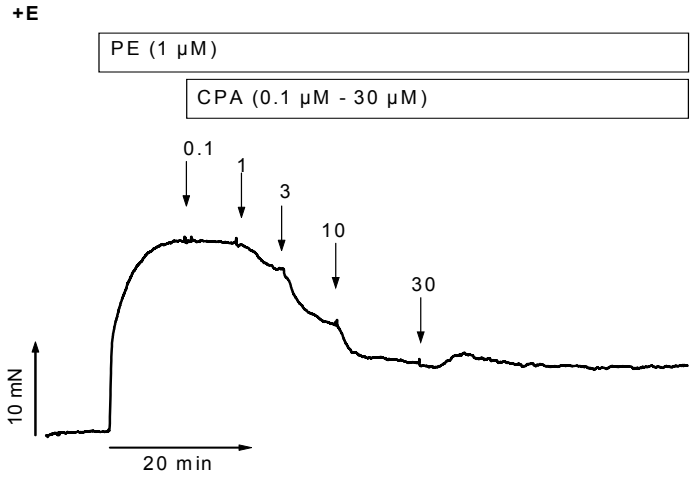

Figure 1. The effects of CPA on PE contractions in endothelium-intact tissues. Experiments were performed in the presence of 10 µM indomethacin. CPA was administrated during the plateau phase of PE precontraction in a cumulative manner (0.1 - 30 µM).

Effects of SERCA blockade in the presence of endothelium

CPA elicited transient contractions in tissues with endothelium (Figure 2A and B, treatment a). The experiment was repeated in the presence of NOS inhibition (L-NAME) to test the possible contribution of NO on biphasic pattern of the CPA response. CPA elicited a drastic contraction following an initial increase in tone in the presence of L-NAME (Figure 2, treatment b). To test the role of endogenous prostanoids in CPA-induced contractions, the same experimental protocol was repeated in the presence of indomethacin. Thus, CPA-induced contractions were significantly inhibited (Figure 2A and B, treatment c).

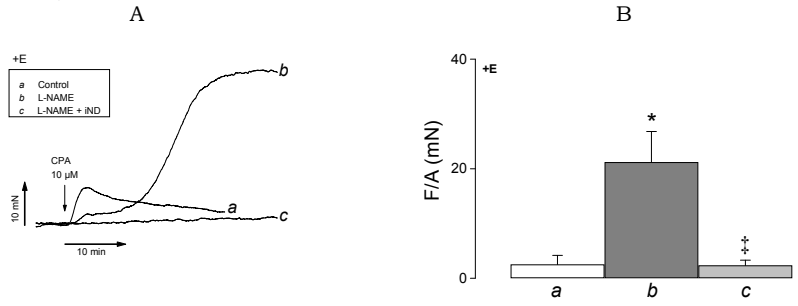

Figure 2. The effects of CPA in the presence of NOS and COX inhibitors in endothelium-intact tissues. A: representative tracings. B: cumulative data. The effects of CPA were tested in a) control conditions, b) 200 µM L-NAME and c) L-NAME plus 10 µM indomethacin. Comparison of CPA responses: *, a vs. b (P<0.05, n=3); ‡, b vs. c (P<0.01, n=3). F/A, force normalized to cross-sectional area.

Effects of SERCA blockade in the absence of endothelium

Similar experimental procedures performed in endothelium-intact rat thoracic aorta rings were repeated in endothelium-denuded aortic rings to investigate the source of contractile and vasorelaxing factors. In the absence of endothelium, CPA yielded drastic and persistent contractions (Figure 3A) similar to that observed in intact tissues with L-NAME treatment (*cf.* Figure 2A and B, treatment b). CPA contractions were enhanced in the presence of L-NAME comparable to that of control (P<0.05, Figure 3A and B, treatment b). During L-NAME and IND treatment, CPA contractions were decreased slightly but significantly (P<0.05, Figure 3A and B, treatment c). In this condition, CPA-induced

contractions were always developed in an oscillatory manner (Figure 3, treatment c).

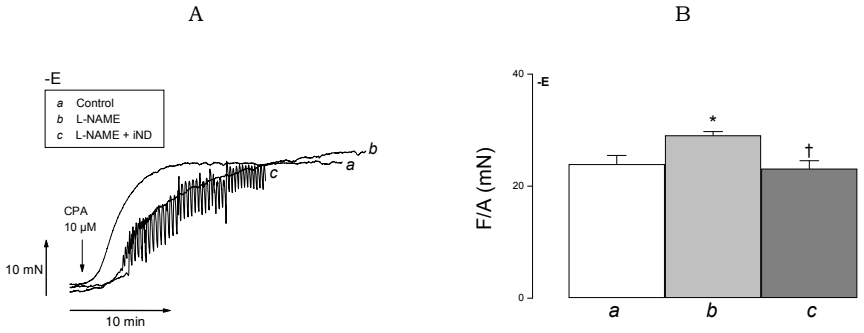

Figure 3. The effects of CPA in the presence of NOS and COX inhibitors in endothelium-denuded tissues. A: representative tracings. B: cumulative data. The effects of CPA were tested in a) control conditions, b) 200 µM L-NAME and c) L-NAME plus 10 µM indomethacin. Comparison of CPA responses: *, a vs. b; †, b vs. c (P<0.05, n=3). F/A, force normalized to cross-sectional area.

Discussion

Present data suggest that vascular effects of SERCA blockade are modulated by NOS and COX enzymes found in both endothelium and vascular smooth muscle.

Effects of SERCA blockade in the presence of endothelium

In endothelium-intact aortic, CPA induced drastic contractions in the presence of L-NAME (present data). However, in intact mouse aorta, only small and transient contraction has been observed[24]. This shows somewhat an apparent discrepancy even between two related species. CPA-induced concentration-dependent relaxations occurred in intact tissues further confirm the presence of tonic inhibitory effects of endothelium over vascular contractions due to SOCE that is activated simultaneously in both cell types[19]. Reversal of CPA-induced relaxations after 10 µM CPA suggests that affinity of endothelial SERCA (SERCA3) to CPA may be higher than that of vascular isoform (SERCA2b)[27].

It has been shown in intact mouse aorta that CPA-induced transient contraction was abolished either by indomethacin or TxA_2 receptor blocker, SQ29548 [24]. This shows that, during eNOS inhibition

in intact aorta, SOCE elevated by SERCA3 inhibition activates endothelial prostanoid (TxA_2/PGH_2) synthesis and release, ultimately leading to vasoconstriction[24]. Caveolar internalization required for endothelin-1 responses in VSMCs[28] is also induced by elevated SOC in endothelial cells. However, in this case internalization of caveola yields removal of Cav-1's inhibition on eNOS ultimately leading to the NOS production and VSMC relaxation[29] (Figure 4).

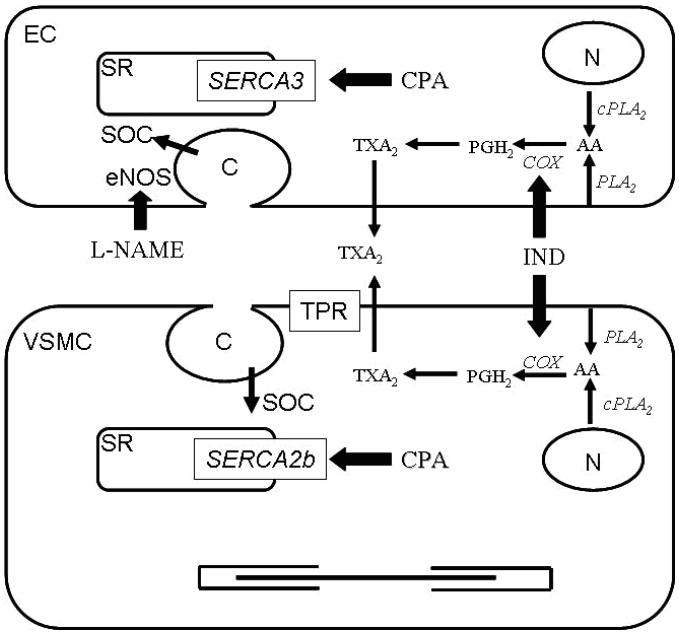

Figure 4. *A model showing the key elements CPA-induced SOCE in endothelial and vascular smooth muscle cells. CPA indiscriminately blocks both endothelial and vascular isoforms of SERCA (SERCA3b and SERCA2b, respectively) leading to depletion of SRs in both cell types. Decrease in Ca^{2+} content of SR activates SOC. Elevation of SOC within subsarcolemmal restricted areas stimulates caveolar internalization removing inhibitory effect of Cav-1 on eNOS (see text for details). In the presence of L-NAME, TxA_2 synthesized from AA of sarcolemmal as well as nuclear envelope origin in either cell types causes IND-sensitive vasocontractions. AA, arachidonic acid; CPA, cyclopiazonic acid; C, caveola; COX: cyclooxygenase; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; IND, indomethacin, N, nucleus; PLA_2, phospholipase A_2; PGH_2, prostaglandin H_2; SOC: store-operated Ca^{2+} channel; SR, sarcoplasmic reticulum; SERCA, SR Ca^{2+} ATPase; TXA_2, tromboxan A_2; TPR, TxA_2 receptor; VSMC, vascular smooth muscle cell. Bold arrows emphasize the inhibitory effects of CPA, L-NAME or IND.*

Effects of SERCA blockade in the absence of endothelium

While no effect observed in endothelium-denuded mouse aorta[24], CPA caused significant contractions in endothelium-denuded rat aorta (present study). On the other hand, enhancement of CPA contractions in the presence of L-NAME suggests that SOCE also activates inducible NOS (iNOS) in VSMCs. Despite the data obtained in endothelium-denuded mouse aorta, IND-resistant CPA-induced contractions in rat aorta may point out species- and age-dependent changes in vascular SERCA (SERCA2b) affinity to CPA[30-32].

Observation of significant CPA contractions during L-NAME and indomethacin treatment in endothelium-denuded aorta further suggest presence of a endothelium-derived vasorelaxing factor that may be lost by endothelium removal (*cf.* treatment c in Figure 2 and 3). In addition, cytosolic PLA_2 ($cPLA_2$) may also contribute to prostanoid synthesis through arachidonic acid production[33]. COX that may be co-localized with $cPLA_2$ which is translocated to nuclear membrane may not be readily accessible by inhibitors due to intracellular diffusion barriers[33] (Figure 4).

In conclusion, any age- or stress-dependent SERCA downregulation may prevent replenishment of agonist-sensitive SRs, a well-established stimulus for SOC activity. In this case, vascular contractions may not be compensated by endothelial-derived relaxing factors in the presence of endothelial dysfunction that possibly worsens the existing vasospastic cases such as hypertension, coronary insufficiency and stroke. Therefore, modulation of SOCE-associated signaling pathways appears to be clinically important. Since there is no conventional SOC inhibitor available, development of *in vivo* molecular intervention methods (i.e. post-transcriptional gene silencing) would be quite promising for prospective treatment of vasospastic disorders.

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Summary

Role of Endothelium on Cyclopiazonic Acid-induced Vascular Contractions in Rat Aorta

This study investigates the mechanism of action of store-operated calcium entry (SOCE) on vascular responses in isolated rat thoracic aorta. For this purpose, effects of cyclopiazonic acid (CPA), a selective blocker of sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) were tested by the use of nitric oxide synthase (NOS) and cyclooxygenase enzyme inhibitors in intact as well as in endothelium-denuded tissues. In intact vessels, the transient contractile response induced by CPA, at concentrations (10 μM) that reportedly deplete the intracellular Ca^{2+} stores, was elevated drastically in the presence of L-NAME whereas it was inhibited by indomethacin. On the other hand, CPA elicited persistent and significant contractions in deendothelialized vessels despite the presence of both enzyme inhibitors. The data show that while CPA-induced store-operated calcium entry stimulates the release of vasoconstricting substances from endothelium and smooth muscle it also reverses the vasoconstriction via activating NOS. In conclusion, aging- or disease-related SERCA down-regulation accompanied by endothelial dysfunction may lead to detrimental vasospasms.

Keywords: store-operated calcium, L-NAME, cyclopiazonic acid, thoracic aorta.

Özet

Sıçan Aortunda Siklopiazonik Asitin Neden Olduğu Vasküler Kontraksiyonlarda Endotelyumun Rolü

Bu çalışmada vasküler düz kas ve endotelyumda depo-kontrollü kalsiyum girişinin (Store-Operated Calcium Entry, SOCE) vasküler yanıtlar üzerindeki etki mekanizması izole sıçan torasik aortunda araştırılmıştır. Bu amaçla selektif sarkoplazmik-endoplazmik retikulum kalsiyum ATPaz (SERCA) inhibitörü siklopiazonik asitin (CPA) etkisi endotel varlığı ve yokluğunda nitrik oksit sentaz (eNOS) ve siklooksijenaz enzim inhibitörleri ile test edilmiştir. Kalsiyum depolarını tamamen boşalttığı bilinen 10 μM CPA'nın endotel varlığında oluşturduğu küçük ve geçici kontraktil yanıt L-NAME varlığında dramatik olarak artarken indometazin varlığında inhibe edilmiştir. Diğer yandan, CPA endotelsiz sıçan aorta halkalarında her koşulda kalıcı ve ileri düzeyde kontraksiyonlara neden olmuştur. Bulgularımız, her iki dokuda da CPA'nın neden olduğu SOCE'nin hem endotel hem de düz kastan vazokonstriktör maddelerin salıverilişini uyarırken diğer yandan NOS'u aktive ederek oluşan kontraksiyonu baskıladığını göstermektedir. Sonuç olarak, yaşlanmaya veya hastalıklara bağlı oluşabilecek SERCA ekspresyonundaki azalmalara endotel hasarının eşlik etmesi istenmeyen düzeyde vazospazmlara neden olabilir.

Anahtar sözcükler: depo-kontrollü kalsiyum, L-NAME, siklopiazonik asit, torasik aort.

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