Resveratrol Stimulates the Na^+–Ca^{2+} Exchanger on the Plasma Membrane to Reduce Cytosolic Ca^{2+} in Rat Aortic Smooth Muscle Cells

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Abstract: Resveratrol is well known to exhibit vascular relaxant and antihypertensive effects. In this study, we determined the effects of resveratrol on the modulation of cytosolic [Ca^{2+}] level and adenosine 5′-triphosphate–induced Ca^{2+} release from the sarcoplasmic reticulum (SR) in rat aortic smooth muscle cells (ASMCs) and explored its underlying mechanisms. In this article, cytosolic [Ca^{2+}] and SR [Ca^{2+}] in ASMCs were determined by Fluo-4/acetoxymethyl and Mag-Fluo-4/acetoxymethyl respectively. Resveratrol (20, 50, and 100 μM) caused a rapid and substantial reduction in cytosolic [Ca^{2+}] in ASMCs bathed in normal Hank's Balanced Salt Solution or Ca^{2+}-free Hank's Balanced Salt Solution. Pretreatment with resveratrol reduced adenosine 5′-triphosphate–induced SR Ca^{2+} release and SR Ca^{2+} content. In the cells bathed in Na^+-free physiological saline, which favors the reverse mode of the Na^+–Ca^{2+} exchanger (NCX), resveratrol induced an increase in cytosolic [Ca^{2+}] and SR [Ca^{2+}]. However, its effect on cytosolic [Ca^{2+}] was inhibited by the selective NCX inhibitor, SEA0400. Our findings suggest that resveratrol reduces cytosolic [Ca^{2+}] and SR [Ca^{2+}] in ASMCs in normal physiological saline, which might be, at least in part, mediated by the NCX.

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INTRODUCTION

Many natural compounds from herbs have beneficial effects on the cardiovascular system.[1,2] Resveratrol (3,4,5-trihydroxystilbene) is a natural polyphenol found in grape skins, peanuts, and red wine that might protect against cardiovascular disease, cancer, and neurodegenerative disease.[3] Resveratrol has antioxidant, anti-inflammatory, anticarcinogenic, and antihypertensive effects.[3] In the vascular system, it acts on multiple targets, including sirtuin-1, endothelial nitric oxide synthase, reactive oxygen species, to elicit its beneficial effects.[3–5]

Ca^{2+} is a versatile second messenger that regulates different physiological processes in many tissues and cells.[6] In the vascular system, an increase in cytosolic [Ca^{2+}] in smooth muscle cells induces vascular contraction, whereas an increase in [Ca^{2+}] in endothelial cells may induce vascular relaxation by stimulating endothelial nitric oxide synthase activity and the subsequent production of nitric oxide. The cytosolic [Ca^{2+}] level itself is regulated by multiple Ca^{2+} channels and Ca^{2+} pumps on the plasma membrane and sarco/endoplasmic/sarcoplasmic reticulum (SR/ER) membrane and the Na^+–Ca^{2+} exchanger (NCX) on the plasma membrane.

The NCX plays a major role in the regulation of cytosolic Ca^{2+} and ER/SR Ca^{2+} in multiple cell types, including smooth muscle cells, cardiomyocytes, and vascular endothelial cells.[7] The predominant isoform of NCXs in vascular smooth muscle cells is NCX type-1 (NCX1).[8,9] NCX catalyzes the countertransport of 3 Na^+ for one Ca^{2+}. Under the physiological Ca^{2+} and Na^+ electrochemical gradient, the NCX primarily operates in the forward mode as a Ca^{2+} extrusion mechanism using the energy of the Na^+ gradient.[7] However, the NCX can also operate in the reverse mode to allow Ca^{2+} influx and Na^+ efflux if the electrochemical gradient of Na^+ and Ca^{2+} favors its reverse operation.[7] Close interactions are known to occur between the plasma membrane and the ER transport systems. The NCX on the plasma membrane not only regulates cytosolic Ca^{2+} levels but also plays a major role in SR/ER Ca^{2+} refilling.[10,11] The forward mode NCX extrudes Ca^{2+} from the cytosol and thus contributes to SR/ER Ca^{2+} unloading/removal, whereas the reverse mode NCX elevates the cytosolic Ca^{2+} level and thus increases SR/ER Ca^{2+} levels.[10–13]

Numerous studies have investigated the effect of resveratrol on the modulation of Ca^{2+} signaling in the cardiovascular system.[5,14,15] However, research on its modulation of Ca^{2+} signaling has derailed because of a major technical problem. Fura-2 is a ratiometric fluorescence dye that is widely considered to be the standard for quantitative cytosolic [Ca^{2+}] measurement.[16] Although Fura-2 is used by many researchers to study the effect of resveratrol on Ca^{2+} signaling,[5,14,17,18] a previous study demonstrated that at concentrations greater than 10 μM resveratrol interferes with Fura-2 cytosolic [Ca^{2+}] measurement, causing severe artifacts.[19] It was shown that resveratrol interacts with Fura-2, causing an increased fluorescence when excited at 340 nm, which is independent of Ca^{2+} change.[19] Interestingly, the same report also showed that resveratrol at 100 μM did not interfere with Fluo-4 cytosolic Ca^{2+} measurements.[19] As a result, many of the previous resveratrol studies that used Fura-2 as the cytosolic [Ca^{2+}] indicator are now controversial.[5,14,17,18] However, even after the exclusion of resveratrol studies that used Fura-2 as the cytosolic [Ca^{2+}] indicator, resveratrol was demonstrated to modulate multiple Ca^{2+} handling processes, including the reduction in basal cytosolic [Ca^{2+}] levels, inhibition of L-type Ca^{2+} current in ventricular myocytes and vascular smooth muscle cells,[14,20] inhibition of the Na^+-H^+ exchanger in cardiomyocytes,[21] and the upregulation of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a in diabetic cardiomyocytes.[22]

In this study, we sought to determine the effects of resveratrol on cytosolic [Ca^{2+}] in primary cultured rat aortic smooth muscle cells (ASMCs) using the single wavelength fluorescence dye, Fluo-4, as the Ca^{2+} indicator. Based on our findings, resveratrol reduces cytosolic [Ca^{2+}] levels and depletes the SR Ca^{2+} stores in ASMCs. Resveratrol may thus act through the NCX to reduce cytosolic [Ca^{2+}] under normal physiological conditions.

METHODS

Animals

Male Sprague–Dawley (SD) rats were obtained from the Laboratory Animal Services Center at the Chinese University of Hong Kong (Hong Kong, China). SD rats were housed in a specific pathogen-free environment with controlled lighting (12:12-hour light–dark cycle). Temperature was maintained at 22–23°C and animals were granted free access to tap water and the standard rodent diet. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, and the procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Cell Culture

The ASMCs were freshly isolated from the thoracic aorta of laboratory male SD rats (age, 7–8 weeks old). After the connective tissue and fat were carefully removed under a microscope, the arteries were cut into small sections under sterile conditions and cultured in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic in a 37°C incubator. The culture medium was replaced every 3–4 days. The ASMCs were grown from the explants (see **Fig. S1A**, **Supplemental Digital Content 1**, http://links.lww.com/JCVP/A511) and achieved confluence after approximately 7 days. Cells were harvested with trypsin and used for experiments at passages 2–3 (see **Fig. S1B**, **Supplemental Digital Content 2**, http://links.lww.com/JCVP/A511).

Ca^{2+} Measurements

Cytosolic Ca^{2+} was measured as previously described.[23] Briefly, ASMCs were loaded with Fluo-4/acetoxymethyl (AM) (5 μM, Invitrogen) or Mag-Fluo-4/AM (5 μM, Invitrogen) or Fura-2/AM (5 μM, Invitrogen) for 30 minutes in the dark at 37°C. Fluo-4 is a high-affinity Ca^{2+} dye that shows a >100-fold increase in fluorescence when bound to Ca^{2+} with excitation wavelength at 488 nm.[24] Fura-2 is a ratiometric high-affinity Ca^{2+} dye with excitation wavelength at 340 and 380 nm. The binding of Fura-2 to Ca^{2+} results in an increased fluorescence when excited at 340 nm and concurrent decrease in fluorescence when excited at 380 nm.[25] Both Fluo-4 and Fura-2 are suitable to measure the cytosolic Ca^{2+} level, which is close to 100 nM. Mag-Fluo-4 is a low-affinity Ca^{2+} indicator suitable to measure high Ca^{2+} concentration level in the endoplasmic reticulum, which is in the range of 100 μM to 1 mM.[26] Fluo-4 or Mag-Fluo-4 was excited at 488 nm and captured at wavelengths of 505–530 nm. Data acquisition was performed with a confocal microscope (Olympus FV1000). Fura-2 was excited by dual excitation wavelength at 340 and 380 nm and captured at wavelength of 510 nm. The ratio of F340/F380 was calculated and acquired with MetaFluor imaging software (Molecular Devices). The Ca^{2+} imaging experiments were performed in Hank's Balanced Salt Solution (HBSS) containing (mM) the following: NaCl 150, KCl 6, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10, adjusted to pH 7.40 with NaOH. Some experiments were performed with Ca^{2+}-free HBSS containing (mM) the following: NaCl 150, KCl 6, $MgCl_2$ 1, glucose 10, and HEPES 10, adjusted to pH 7.40 with NaOH. For the Na^+-free solution, Na^+ was replaced with N-methyl-D-glucamin in normal HBSS. The amplitude of Ca^{2+} was displayed as a ratio of the maximal/minimal fluorescence relative to the basal intensity (F1/F0) for Fluo-4/Mag-Fluo-4 or change of 340/380 ratio for Fura-2. All experiments were performed at room temperature.

Resveratrol (20, 50, and 100 μM) were added in normal HBSS or Ca^{2+}-free HBSS. In resveratrol pretreatment experiments, the cells were pretreated with resveratrol for 10 minutes before 100 μM adenosine 5′-triphosphate (ATP) or 5 μM thapsigargin challenge. When ATP-induced Ca^{2+} transient returned to baseline, 5 μM ionomycin was added to induce full SR Ca^{2+} release independent of inositol 1,4,5-trisphosphate receptor (IP3R). In SEA0400 experiments, the cells were pretreated with SEA0400 for 10 minutes before 50 μM resveratrol challenge.

Isometric Tension Measurement

Segments of C57/BL6 mouse aorta ~2 mm in length were dissected from ~5-week-old male C57BL/6 mice, and the endothelial layer was rubbed off. The segments were

mounted in a wire myograph (610M; Danish Myograph Technology) under a normalized tension as previously described.[27] The aortic segments were preconstricted with 10 μM phenylephrine to achieve sustained contractions. Resveratrol was added in a cumulative fashion to the bath solution, which was Krebs solution containing the following, in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.2, and glucose 11.1, gassed with 95% O_2–5% CO_2. Experiments were performed at 37°C. Data were acquired and analyzed using PowerLab and LabChart (ADInstruments).

Reagents

Stock solutions of trans-resveratrol (Sigma), thapsigargin (Sigma), ionomycin (Sigma), and SEA0400 (Sigma) were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.1% in each experiment. ATP disodium salt hydrate (Sigma) was prepared in H_2O. U46619 (Sigma) was dissolved in methyl acetate. All other chemicals were purchased from Sigma.

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). The data for each condition were derived from at least 4 independent experiments. Statistical significance between 2 groups was obtained by Student's *t* test. Groups of 3 or more were analyzed using one-way analysis of variance, followed by Bonferroni post hoc test. Statistical analyses were performed with Prism 6.0 software (GraphPad Software Inc, La Jolla, CA). A P value of <0.05 was considered to indicate statistical significance.

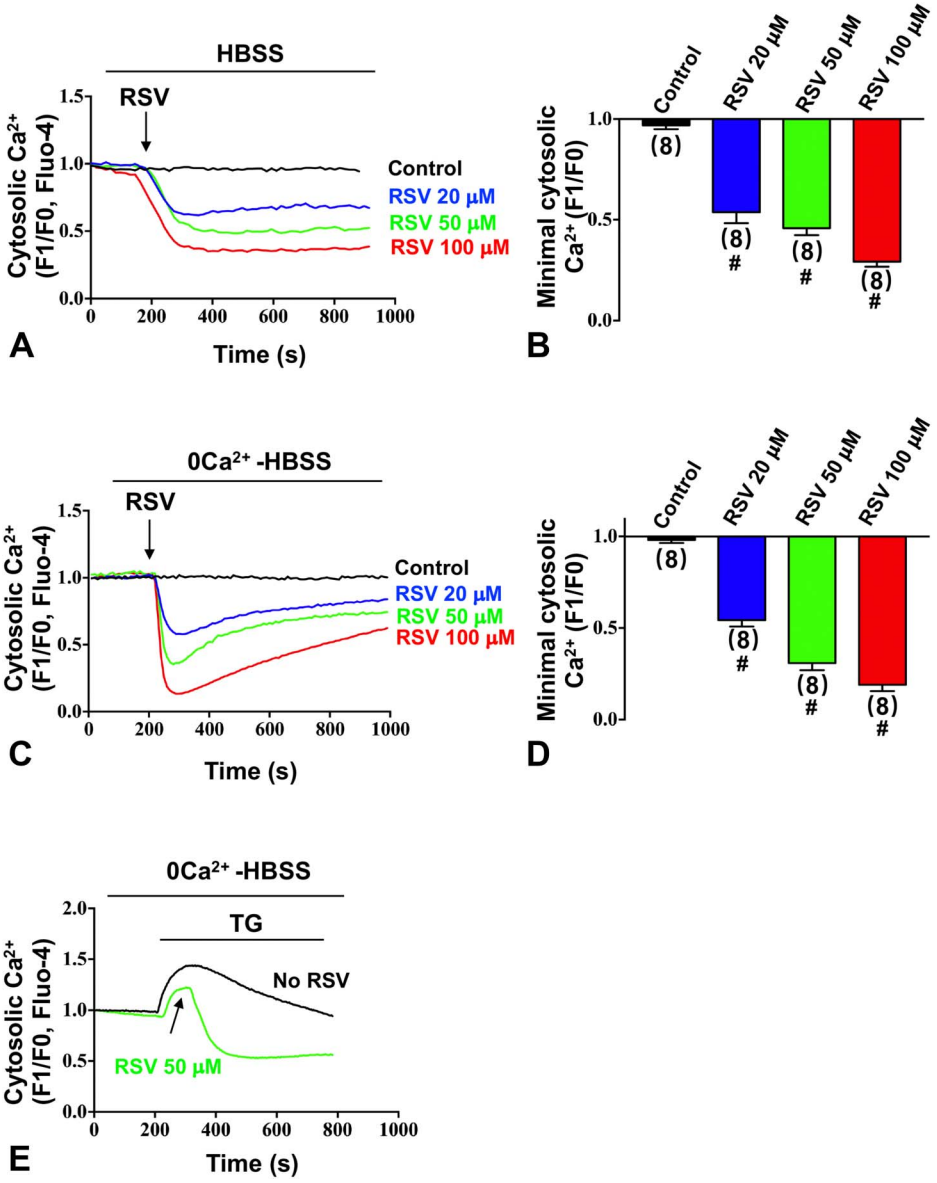

FIGURE 1. Resveratrol induced a reduction in cytosolic [Ca^{2+}]. A and C, Representative time course traces of cytosolic [Ca^{2+}] in response to resveratrol in ASMCs. The cells were challenged with resveratrol (20, 50, and 100 μM) in HBSS (A) or Ca^{2+}-free HBSS (C). B and D, Data summary of the maximal cytosolic [Ca^{2+}] reduction in response to resveratrol as in (A) and (C). E, Representative time course traces of cytosolic [Ca^{2+}] in response to 5 μM of thapsigargin (TG), followed by 50 μM of resveratrol in cells bathed in Ca^{2+}-free HBSS. The arrow indicates the time point when resveratrol was applied. The control was not challenged with resveratrol. Values in the data summary are expressed as mean ± SEM (n = 8 experiments). Each n represents one experiment performed using ASMCs from one individual rat, containing 10–15 cells. #$P < 0.001$ compared with the control. n = 4 in (E).

RESULTS

Resveratrol Decreased Cytosolic [Ca^{2+}] in ASMCs

The primary cultured ASMCs were loaded with 5 μM Fluo-4, a Ca^{2+}-sensitive fluorescence indicator. Thereafter, the cells were bathed in normal physiological saline, HBSS. The application of trans-resveratrol in the concentration range, 20–100 μM, caused a concentration-dependent reduction in cytosolic [Ca^{2+}] (Figs. 1A, B and see **Fig. S2**, **Supplemental Digital Content 3**, http://links.lww.com/JCVP/A512). Also, when cells were bathed in Ca^{2+}-free HBSS, resveratrol could decrease cytosolic [Ca^{2+}] (Figs. 1C, D). The effect of resveratrol on cytosolic [Ca^{2+}] seemed to be stronger in cells bathed in Ca^{2+}-free HBSS than in cells bathed in normal HBSS (Figs. 1A, C). One likely explanation is that, in normal HBSS, Ca^{2+} entry through the plasma membrane partially offset the cytosolic Ca^{2+} reduction.

In the cells bathed in Ca^{2+}-free HBSS, the application of 5 μM thapsigargin induced an increase in cytosolic [Ca^{2+}] (Fig. 1E); this was an expected response owing to the inhibition of the SR Ca^{2+} pump by thapsigargin. In the presence of 5 μM thapsigargin, resveratrol could still reduce cytosolic [Ca^{2+}] (Fig. 1E).

A previous study showed that resveratrol may interact with Fura-2 to cause Fura-2 fluorescence increase when excited at 340 nm, which is independent of Ca^{2+}. Indeed, our results confirmed that 100 μM resveratrol induced Fura-2 fluorescence increase when excited at 340 nm (see **Fig. S3**, **Supplemental Digital Content 4**, http://links.lww.com/JCVP/A513).

Pretreatment With Resveratrol Reduced ATP-induced and Thapsigargin-induced Cytosolic [Ca^{2+}] Increase Through Depleting the SR Ca^{2+} Stores

The major SR Ca^{2+} release channel in ASMCs is the IP3R on the SR.[29] Here, ATP was used to induce IP3R-mediated Ca^{2+} release from the SR. The primary cultured ASMCs were bathed in Ca^{2+}-free HBSS and pretreated with or without resveratrol for 10 minutes. In the control cells that were not pretreated with resveratrol, the application of ATP (100 μM) caused a rapid and transient increase in cytosolic [Ca^{2+}] (Fig. 2A). However, pretreatment with resveratrol nearly abolished the ATP-induced increase in cytosolic [Ca^{2+}] (Figs. 2A, B). A reduced cytosolic [Ca^{2+}] response to ATP could result from a low SR Ca^{2+} content or inhibition of IP3R on the SR membrane. To differentiate between these 2 probabilities, we used ionomycin-induced Ca^{2+} release as an indicator of SR Ca^{2+} content. Ionomycin is a Ca^{2+} ionophore that can cause full release of SR Ca^{2+} independent of IP3R function.[29] Treatment with resveratrol reduced the subsequent SR Ca^{2+} release to 5 μM ionomycin, confirming that the resveratrol treatment reduced the SR Ca^{2+} content (Figs. 2A, B).

Resveratrol pretreatment for 10 minutes decreased the magnitude of the thapsigargin-induced cytosolic [Ca^{2+}] increase in ASMCs bathed in Ca^{2+}-free HBSS (Figs. 2C, D), further confirming that resveratrol can reduce the SR Ca^{2+} content. The subsequent re-addition of Ca^{2+} caused a second cytosolic [Ca^{2+}] increase (Fig. 2C), which represented the store-controlled entry of Ca^{2+}. However, the magnitude of the store-controlled Ca^{2+} entry was reduced by pretreatment with resveratrol (Figs. 2C, D).

To verify that resveratrol could indeed reduce the SR Ca^{2+} content, the primary cultured ASMCs in Ca^{2+}-free HBSS were loaded with a low-affinity Ca^{2+} fluorescence dye, Mag-fluo-4, which preferentially labels SR [Ca^{2+}].[30] The application of resveratrol caused a rapid reduction in SR [Ca^{2+}] in a dose-dependent manner (Figs. 2E, F).

Resveratrol-stimulated NCX

We proceed to investigate the potential involvement of the NCX, a major mechanism for Ca^{2+} extrusion from cells.[7] The primary cultured ASMCs were bathed in Na^{+}-free HBSS, which favors the reverse mode of NCX. Interestingly, under this condition, resveratrol caused an increase in cytosolic [Ca^{2+}] (Fig. 3A) and SR [Ca^{2+}] (Fig. 3B), suggesting that resveratrol stimulated the activity of NCX.

Next, we used SEA0400, a selective inhibitor of NCX. The primary cultured ASMCs were bathed in Ca^{2+}-free HBSS, which favors the forward mode of the NCX. The application of 50 μM of resveratrol caused a rapid reduction in cytosolic [Ca^{2+}] (Fig. 3C), which was reduced by pretreatment with 2 μM of SEA0400 (Figs. 3C, D).

Resveratrol-induced Vascular Dilation

To demonstrate the physiological relevance of resveratrol at the concentration we used, we examined the effect of resveratrol in the concentration range of 10–100 μM on vascular tone. The results showed that resveratrol of 50 and 100 μM caused dose-dependent relaxation in mouse aortas preconstricted with 100 nM U46619 (Fig. 4).

DISCUSSION

Based on the results presented in this article, resveratrol (1) induced a dose-dependent reduction in cytosolic [Ca^{2+}] and SR [Ca^{2+}] levels in ASMCs bathed in normal physiological saline or Ca^{2+}-free HBSS; (2) reduced SR Ca^{2+} release in response to ATP and thapsigargin in ASMCs, reduced SR [Ca^{2+}] levels as demonstrated by the direct measurement of Mag-Fluo-4 fluorescence, and decreased store-operated Ca^{2+}entry; and (3) induced an increase in cytosolic [Ca^{2+}] and SR [Ca^{2+}] when cells were bathed in Na^{+}-free physiological saline, which favors the reverse mode of NCX. However, the effect of resveratrol on the reduction in cytosolic [Ca^{2+}] was inhibited by the selective NCX inhibitor, SEA0400. Altogether, our findings suggest that in vascular smooth muscle cells, resveratrol decreases cytosolic [Ca^{2+}] and SR [Ca^{2+}] levels, at least in part, by stimulating the NCX.

The application of resveratrol was found to cause instantaneous and substantial reductions in basal cytosolic [Ca^{2+}] and SR [Ca^{2+}] in ASMCs. Furthermore, in the ASMCs pretreated with resveratrol, SR Ca^{2+} release in response to ATP (a Ca^{2+} mobilizing agent) was reduced, which might

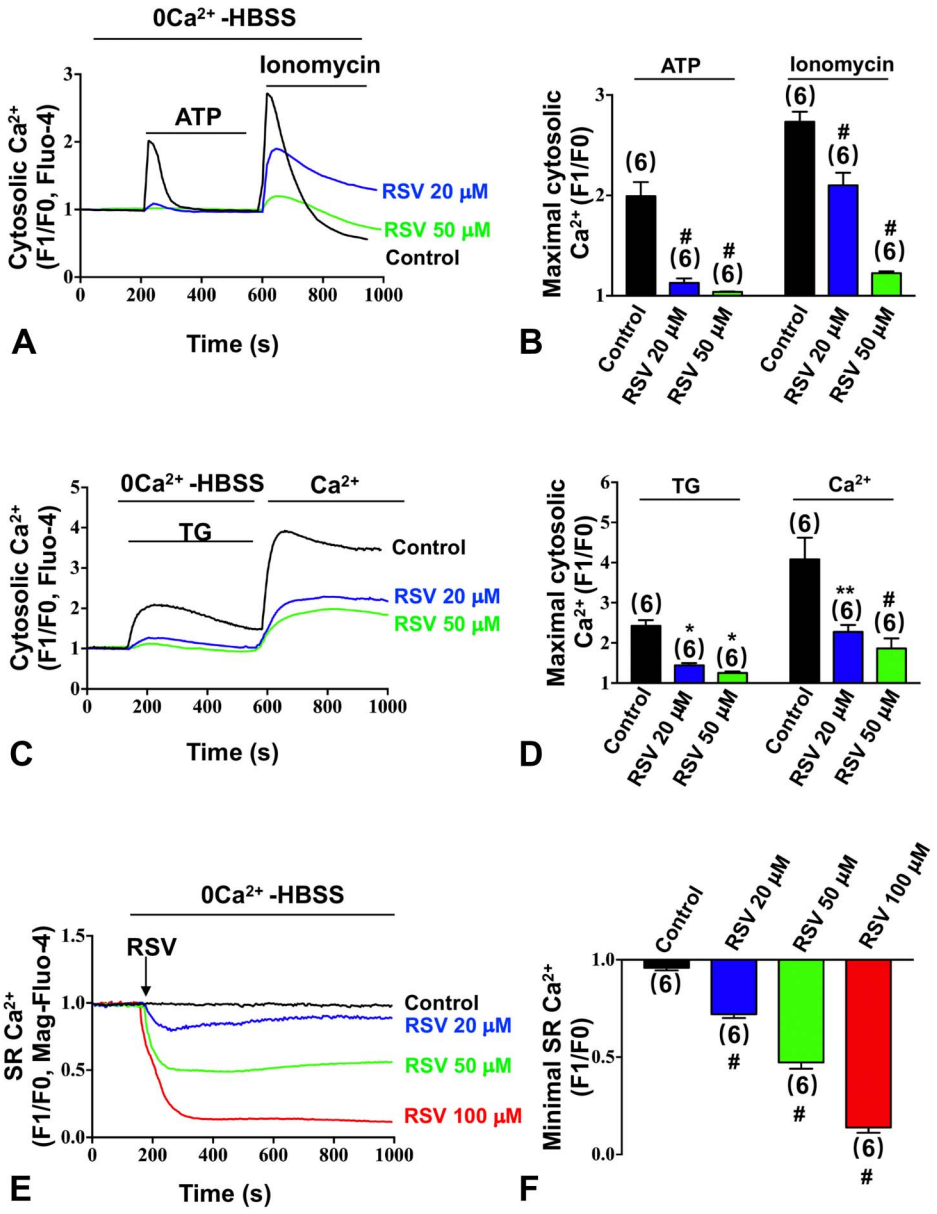

FIGURE 2. Resveratrol pretreatment reduced the ATP-induced and thapsigargin (TG)-induced cytosolic [Ca^{2+}] increase by depleting the SR Ca^{2+} stores. ASMCs were bathed in Ca^{2+}-free HBSS. A–D, Representative time course traces (A and C) and data summary (B and D) of cytosolic [Ca^{2+}] in response to 100 μM of ATP and 5 μM of ionomycin (A and B) or 5 μM of thapsigargin, followed by the re-addition of 1.5 mM Ca^{2+} (C and D). The ASMCs in Ca^{2+}-free HBSS were preincubated with resveratrol (20 and 50 μM, 10 minutes) before ATP or thapsigargin challenge. Control cells were not pretreated with resveratrol. E and F, The time course traces (E) and data summary (F) of SR [Ca^{2+}] using Mag-Fluo-4. The cells in Ca^{2+}-free HBSS were challenged with resveratrol (20, 50, and 100 μM). Values in the data summary are expressed as mean ± SEM (n = 6 experiments). Each n represents one experiment performed using ASMCs from one individual rat, containing 10–15 cells. *$P < 0.05$, **$P < 0.01$, #$P < 0.001$ compared with the control.

be a consequence of SR Ca^{2+} store depletion. Although these results align with those published for other cell types, including cardiomyocytes and Hela cells,[31,32] they contradict those of several studies that reported an increase in cytosolic [Ca^{2+}] in vascular cells by resveratrol through Fura-2 fluorescence.[5,17] The findings of the previous studies might be due to the artifact resulted from interaction between resveratrol and Fura-2 independent of Ca^{2+} or cell types,[5,17] which has been reported and well discussed in a previous study[19] and also confirmed by us in this study.

Notably, the ability of resveratrol to reduce basal cytosolic [Ca^{2+}] levels and agonist-induced SR Ca^{2+} release may have important functional implications in arterial smooth muscle cells. Previous studies found that resveratrol can not only induce endothelium-dependent vascular relaxation but also cause substantial endothelium-independent relaxation in many arterial types.[33–35] We also confirmed that resveratrol at the concentration of 50 and 100 μM could induce dose-dependent relaxation in mouse aorta. Based on the results from this study, we speculate that the well-documented vascular relaxant and antihypertensive effects of resveratrol could be due to a reduction in basal cytosolic [Ca^{2+}] and agonist-induced SR Ca^{2+} release in vascular smooth muscle cells by resveratrol.[4]

In this article, we explored the mechanism that might be used by resveratrol to reduce cytosolic [Ca^{2+}] and SR [Ca^{2+}]. Because resveratrol induced a rapid reduction in cytosolic [Ca^{2+}], even in the absence of extracellular [Ca^{2+}], this reduction in cytosolic [Ca^{2+}] must be due to the stimulation of a cytosolic Ca^{2+} removal mechanism. These mechanisms may include enhanced Ca^{2+} extrusion through the plasma membrane using the NCX or stimulated Ca^{2+} sequestration to the

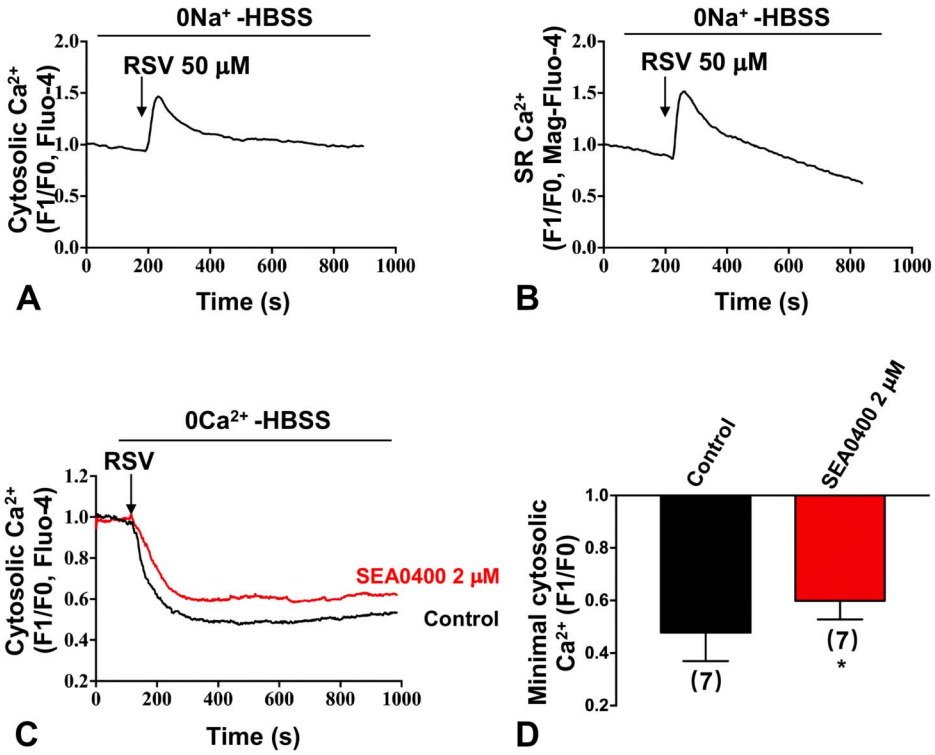

FIGURE 3. Resveratrol stimulated the NCX. A and B, Representative time course traces of cytosolic [Ca^{2+}] (A) and SR [Ca^{2+}] (B) in response to the challenge with 50 μM of resveratrol in ASMCs bathed in Na^+-free physiological saline. C and D, Representative traces (C) and data summary (D) of cytosolic [Ca^{2+}] in response to 50 μM of resveratrol. The cells bathed in Ca^{2+}-free HBSS were pretreated with or without SEA0400 (2 μM, 30 minutes). Values in the data summary are expressed as mean ± SEM (n = 7 experiments). Each n represents one experiment performed using ASMCs from one individual rat, containing 10–15 cells. *$P < 0.05$ compared with the control.

SR Ca^{2+} stores through SR Ca^{2+}-ATPase. However, resveratrol was found to reduce cytosolic [Ca^{2+}] levels even when the SR ATPase was inhibited by thapsigargin (Fig. 1E), suggesting that the reduction in cytosolic [Ca^{2+}] by resveratrol was not caused by stimulated Ca^{2+} sequestration to SR Ca^{2+} stores. Indeed, direct measurement of SR [Ca^{2+}] concentration revealed that resveratrol treatment decreased rather than increased the SR [Ca^{2+}] level, further supporting the finding that resveratrol did not stimulate cytosolic Ca^{2+} sequestration to SR Ca^{2+} stores. Therefore, the reduction in cytosolic [Ca^{2+}] by resveratrol may be due to its promotion of Ca^{2+} extrusion through the plasma membrane. We determined the role of the NCX, a major player in Ca^{2+} extrusion across the plasma membrane under the physiological Ca^{2+} and Na^+ electrochemical gradient.[7] Interestingly, when the cells were bathed in normal physiological saline, which favors the forward mode of the NCX, resveratrol caused a reduction in cytosolic [Ca^{2+}]. However, when the cells were bathed in Na^+-free physiological saline, which favors the reverse mode of the NCX, resveratrol induced an increase in cytosolic [Ca^{2+}]. The ability of resveratrol to reduce cytosolic [Ca^{2+}] was found to be inhibited by the selective NCX inhibitor, SEA0400. Therefore, these outcomes suggest that resveratrol might, at least in part, act through the NCX to reduce cytosolic [Ca^{2+}] in ASMCs. Furthermore, because the onset of resveratrol effect on [Ca^{2+}] was very rapid, it was unlikely caused by change in the NCX expression level. Instead, a more likely possibility is that resveratrol may directly act on NCX to stimulate its activity. Previously, resveratrol has been reported to interact directly with other ion transport protein, such as ryanodine receptors.[36]

Based on our findings, resveratrol caused a concomitant increase/decrease in cytosolic [Ca^{2+}] and SR [Ca^{2+}]. In response to resveratrol challenge, cytosolic [Ca^{2+}] and SR [Ca^{2+}] either concomitantly increased or decreased. These results align with the notion that the forward mode NCX

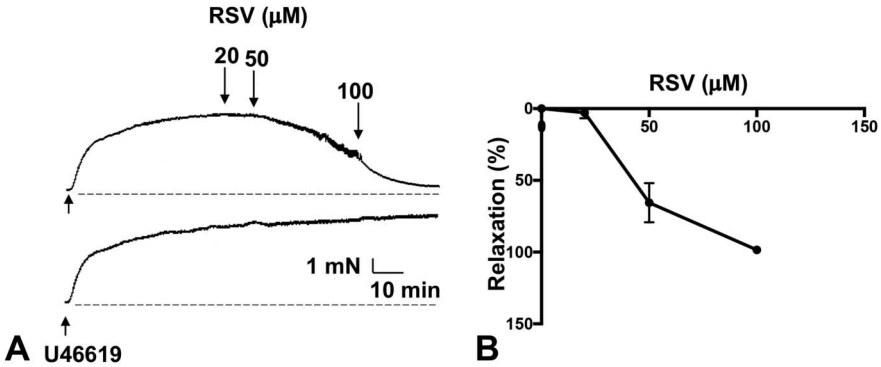

FIGURE 4. The vascular relaxant effect of resveratrol on 100 nM U46619 precontracted mouse aortas. Shown are representative time courses of isometric tension in isolated C57 mouse aortic segments in response to cumulatively increasing concentrations of resveratrol (A, upper panel) applied to the bath. A time control trace without resveratrol application is shown in (A, lower panel). Data summary are shown in (B). The vessels were preconstricted with 100 nM U46619 (n = 5 experiments).

simultaneously decreases cytosolic [Ca^{2+}] and ER [Ca^{2+}], whereas the reverse mode NCX concomitantly increases cytosolic [Ca^{2+}] and ER [Ca^{2+}].[10–13] Therefore, in normal physiological saline, resveratrol may stimulate NCX in the forward mode to facilitate cytosolic Ca^{2+} removal resulting in a reduction in basal cytosolic [Ca^{2+}]. Although the data obtained under our experimental conditions strongly suggested that resveratrol regulated NCX1 channel activity, patch clamp experiments would strengthen this conclusion.[37]

Previously, resveratrol was demonstrated to inhibit L-type Ca^{2+} channels in vascular smooth muscle cells.[14] In this article, resveratrol was found to decrease the store-operated Ca^{2+} entry (Figs. 2C, D). Therefore, when vascular smooth muscle cells are bathed in a normal Ca^{2+}-containing physiological medium, resveratrol may reduce cytosolic [Ca^{2+}] and SR [Ca^{2+}] through multiple mechanisms, including the stimulation of the NCX, inhibition of L-type Ca^{2+} channels, and reduction of store-controlled Ca^{2+} entry.

CONCLUSION

In conclusion, the findings of this study suggest that under physiological conditions, resveratrol decreases basal cytosolic [Ca^{2+}] levels, attenuates the agonist-stimulated [Ca^{2+}] increase, and reduces SR [Ca^{2+}] levels in vascular smooth muscle cells. These actions may be, at least in part, mediated by the stimulation of NCX and reduction of store-controlled Ca^{2+} entry. The effects exhibited by resveratrol on vascular smooth muscle cells may underlie its well-documented vascular relaxant and antihypertensive effects.

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