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# *Trans*- and *cis*-resveratrol increase cytoplasmic calcium levels in A7r5 vascular smooth muscle cells

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The effects of *trans*- and *cis*-resveratrol on cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) were studied using fura-2 in vascular smooth muscle cells (A7r5). Both isomers of resveratrol caused a sustained elevation in $[Ca^{2+}]_i$, *cis*-resveratrol being significantly more effective than the *trans*-isomer. The resveratrol-induced increase in $[Ca^{2+}]_i$ was significantly potentiated by the previous application of low concentrations of thapsigargin, partially inhibited by nifedipine or $Ni^{2+}$, and not affected by SKF 96365. In the absence of extracellular $Ca^{2+}$, both isomers of resveratrol induced a transient, slow increase in $[Ca^{2+}]_i$, which was inhibited by the previous depletion of intracellular stores with thapsigargin and completely blocked by preincubation with TMB-8, an inhibitor of intracellular calcium release. Reintroduction of $Ca^{2+}$ in the external solution after the resveratrol-induced release of $Ca^{2+}$ activated the $Ca^{2+}$ influx through store-operated calcium channels. The resveratrol-induced increase in $[Ca^{2+}]_i$ in the absence of extracelullar $Ca^{2+}$ partially reduced the increase in $[Ca^{2+}]_i$ evoked by the subsequent application of thapsigargin. Our results suggest that *trans*- and *cis*-resveratrol induce a depletion of $Ca^{2+}$ from the same intracellular stores released by thapsigargin and subsequent capacitative influx of $Ca^{2+}$. Additionally, a direct activation of transmembrane $Ca^{2+}$ influx through another type of channel may be also implicated.

**Keywords:** A7r5 / Calcium / Fura-2 / Resveratrol / Thapsigargin

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## 1 Introduction

Resveratrol (3,4′,5-trihydroxystilbene, RESV) is a natural phenolic component of *Vitis vinifera* L. (Vitaceae), abundant in the skin of grapes and present in wines, especially red wines. RESV is not unique to *Vitis* but is also present in at least 72 other plant species (distributed in 12 families and 31 genera; *e.g.*, *Veratrum*, *Arachis*, *Morus*, and *Trifolium*), some of which are components of the human diet, such as mulberries and peanuts. In wines, RESV exits as *cis*- and *trans*-isomers [1, 2] (Fig. 1). To date most of the studies on the biological effects of RESV have considered the *trans*-isomer (*t*-RESV), which has shown a wide range of pharmacological actions including anti-inflammatory, anticarcinogenic, antioxidant, estrogenic, platelet antiaggregatory, and antimicrobial properties (for reviews, see *e.g.* [2–4]). Some of these activities have been implicated in the cardiovascular protective effects attributed to *t*-RESV and red wine [5, 6].

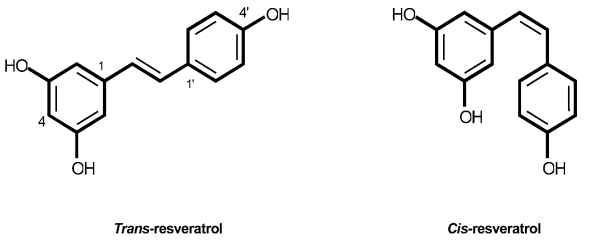


**Figure 1.** Chemical structures of *t*-RESV (3,4′,5-trihydroxy-*trans*-stilbene) and *c*-RESV (3,4′,5-trihydroxy-*cis*-stilbene).

Despite the above described considerations, the *in vitro* vasodilator activity of *t*-RESV in rat aorta has surprisingly received little research attention. Thus, Fitzpatrick *et al*. [7] have reported that *t*-RESV (at concentrations up to 0.1 mM) is unable to relax rat aortic rings, whereas Chen and Pace-Asciak [8] have reported that *t*-RESV (>30 μM) induces relaxation of phenilephrine-precontracted endothelium-intact rat aorta, and at higher concentrations (> 60 μM) also

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**Abbreviations: *c*-RESV**, *cis*-resveratrol; **fura-2 AM**, fura-2 acetoxymethyl ester; **SKF 96365**, 1-(β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride; **SOCC**, store-operated calcium channel; ***t*-RESV**, *trans*-resveratrol; **THAPS**, thapsigargin; **TMB-8**, 3,4,5-trimethoxybenzoic acid 8-diethylamino-octyl ester 8

induces the relaxation of endothelium-denuded rat aorta. Similarly, we have reported that *t*-RESV exhibits in rat aorta: (i) a characteristic endothelium-dependent vasorelaxant effect which seems to be due to the inhibition of vascular NADH/NADPH oxidase and the subsequent decrease of basal cellular superoxide radicals ($O_2^{\bullet -}$) generation and, therefore, of nitric oxide (NO) biotransformation [5] and (ii) an endothelium-independent vasodilator effect, which appears to be mediated by an inhibition of protein kinase C (PKC) [9]. The endothelium-dependent component is probably the most relevant, since it is observed at lower drug concentrations (< 10 μM) than the endothelium-independent effect (> 50 μM).

Much less is known about the pharmacological activity of the *cis*-isomer (*c*-RESV) possibly as a result of the fact that this isomer (unlike *t*-RESV) is not commercially available. *c*-RESV significantly attenuates several components of the macrophage response to proinflammatory stimuli [10], and a few studies have demonstrated only quantitative differences in the activity of the two isomers; for example, in their ability to decrease the collagen-induced platelet aggregation [11], in their estrogenic/antiestrogenic and scavenging properties [12], or in the cyclooxygenase-1 assay [13].

Vascular tone directly depends on cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) levels in smooth muscle cells. Thus, any action of RESV on calcium signaling in these cells may be related to its endothelium-independent vascular actions. Again, there is little information on this subject. Orallo and Camiña [9], using $^{45}Ca^{2+}$ in rat aortic rings, have suggested that the vasorelaxant effect of *t*-RESV is not mediated by blockage of $Ca^{2+}$ influx through transmembrane receptor-operated calcium channels, Rotondo *et al*. [14] reported a *t*-RESV-associated inhibition of agonist-induced $[Ca^{2+}]_i$ increases in polymorphonuclear leukocytes, and Dobrydneva *et al*. [15, 16] described that *t*-RESV inhibits $Ca^{2+}$ influx in thrombin-stimulated human platelets.

Bearing in mind the above considerations and with the aim of providing new data on the effects of RESV on vascular smooth muscle cells, we here report the first detailed comparative study of the effects of both *t*-RESV and *c*-RESV on calcium signaling in single A7r5 cells (a cell line derived from fetal rat aorta), hoping to clarify how RESV affects smooth muscle cell $[Ca^{2+}]_i$ levels.

## 2 Materials and methods

### 2.1 Cell culture

A7r5 smooth muscle cells, a well-established vascular smooth muscle cell line obtained from embryonic rat aorta, were obtained from the American Type Culture Collection (CRL1446; Rockville, MD, USA). The cells were grown in DMEM/F-12 (1 : 1) supplemented with heat-inactivated fetal bovine serum (10%), L-glutamine (2 mM), penicillin G (100 U · $mL^{-1}$), streptomicin (100 μg $mL^{-1}$), and amphotericin B (0.25 μg · $mL^{-1}$). Cells were kept in culture at 37°C with 5% $CO_2$ in air. The cells were subcultured in 75 $cm^2$ tissue culture flasks and, for experiments, in 35 mm Petri dishes in which a 20 mm diameter hole had been cut in the base and replaced by a thin (0.1 mm) glass coverslip. Cells were seeded at low density (~1500 cells $cm^{-2}$) and allowed to grow for at least 24 h in culture medium (see above). Cells were kept in culture for 2 – 5 days before the experiments.

### 2.2 Measurement of $[Ca^{2+}]_i$ in isolated vascular smooth muscle cells

A7r5 cells (plated on Petri dishes as described above) were incubated for 60 min at 37°C in normal bathing solution (composition in mM: NaCl 140, KCl 5, $CaCl_2 \cdot 2H_2O$ 1.5, $MgCl_2$ 2, HEPES 10, glucose 11, pH 7.4) containing 2.5 μM fura-2 acetoxymethyl ester (fura-2 AM), then gently washed twice with normal bathing solution and allowed to rest for >15 min in the incubator, to allow the hydrolysis of fura-2/AM into $Ca^{2+}$-sensitive free acid form by cell esterases. Cells were placed on an inverted microscope (Zeiss Axiovert 135) and continuously superfused at 0.5 mL · $min^{-1}$ with normal bathing solution except during the application of drugs. For experiments in extracellular $Ca^{2+}$-free medium, $CaCl_2$ was replaced by 10 mM EGTA. Measurements were made from isolated cells or small groups of dispersed cells (up to 8 cells) viewed with a 63 × oil immersion objective (Zeiss Plan-Neofluar, 63 × /1.25 oil) using a digital imaging system. Fura-2 was excited alternately at 340 ± 10 and 380 ± 10 nm (100 ms exposure time for both wavelenghts) using a 75 W Xenon lamp (XBO 75W/2). Excitation light from the monochromator (P130/MLE/400 Optoscan; Cairn Research, UK) was reflected off a 400 nm dichroic mirror. Emitted fluorescence was collected through a 510 ± 20 nm emission filter and measured with an intensified CCD camera (C651-ICCD; Cairn Research). Ratiometric $Ca^{2+}$ images were generated at 2 – 20 s intervals, using 4 averaged images at each wavelength. Images were digitally stored and analyzed using MetaFluor software (Universal Imaging Corporation, USA). The short exposition to the 340/380 nm never induced a partial isomerization of *t*-RESV to *c*-RESV. Drugs (or vehicles, for the corresponding control experiments) were added in volumes of 10 – 50 μL to a final incubation volume of 2 mL of bathing solution. All procedures and experiments were performed at room temperature (~20°C) to minimize compartmentalization and cell extrusion of the fluorescent dye.

 

### 2.3 Drugs and chemicals

Amphotericin B, DMSO, *t*-RESV, ionomycin, nifedipine, penicillin G, 3,4,5-trimethoxybenzoic acid 8-diethylaminooctyl ester 8 (TMB-8), 1-(β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride (SKF 96365), and streptomycin were purchased from Sigma (Alcobendas, Spain). Thapsigargin (THAPS) was from RBI (Natick, MA, USA). DMEM/F-12 and fetal bovine serum were from Gibco (Barcelona, Spain). Fura-2 AM was from Molecular Probes (Madrid, Spain). All other reagents were of analytical grade. *c*-RESV was prepared at the Departamento de Química Orgánica (Universidad de Santiago de Compostela) following the method previously described [10]. Stock solutions of these compounds were prepared and stored at –20°C as follows: SKF 96365 (10 mM) and TMB-8 (10 mM) in distilled water; ionomycin (1 mM), nifedipine (10 mM), *t*-RESV (100 mM), *c*-RESV (100 mM), and THAPS (1 mM) in DMSO. From these stock solutions, dilutions in physiological buffer were freshly prepared every day as were needed for experiments. The final concentration of DMSO never exceeded 0.01%. Fura-2-AM (2.5 μM) was prepared daily in physiological buffer containing 0.1% DMSO. Appropriate precautionary measures were taken throughout the procedure to avoid degradation of light-sensitive compounds (*t*-RESV, *c*-RESV, and nifedipine) and extensive photobleaching due to the photosensitivity of the fura-2 molecule.

### 2.4 Data presentation and statistical analysis

Unless otherwise specified, results shown in the text and figures are expressed as means ± SEM. Significant differences between two means ($p < 0.05$ or $p < 0.01$) were determined by Student's two-tailed *t*-test for paired or unpaired data, where appropriate.

For each cell, $[Ca^{2+}]_i$ was averaged from pixels within manually outlined cell areas. Background compensation was performed by substracting the illumination from an area of the image which contained no cells. $[Ca^{2+}]_i$ was then calculated from the 340/380 nm fluorescence ratio as described by Grynkiewicz *et al*. [17]. Ratios were converted to free $Ca^{2+}$ by the equation: $[Ca^{2+}]_i = K_d\ (R - R_{min}/R_{max} - R)\ (F_{min380}/F_{max380})$, where $K_d$ is the affinity constant of fura-2 for $Ca^{2+}$, $R$ is the 340/380 nm fluorescence ratio, $R_{min}$ and $R_{max}$ are the limiting ratios for minimal (0 mM $Ca^{2+}$ and 10 mM EGTA in the bath) and saturating $[Ca^{2+}]_i$ (10 mM $Ca^{2+}$ in the bath) both in the presence of 10 μM ionomycin. $F_{min380}/F_{max380}$ is the ratio of fluorescence of fura-2 at 380 nm in the presence of minimal to that of saturating $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ was determined by averaging resting $Ca^{2+}$ values measured for 10 s on cells from different preparations. Only data obtained from cells that responded to the $Ca^{2+}$ ionophore ionomycin (0.5 μM), in the presence of 1.5 mM of external $CaCl_2$, at the end of the experiments were used. From the cumulative-response curves, the 50% efficacy concentration ($EC_{50}$) for the effect of *t*-RESV or *c*-RESV was calculated using a sigmoidal curve-fitting analysis program (Origin 6.0).

## 3 Results

### 3.1 Effects of *t*-RESV and *c*-RESV on basal $[Ca^{2+}]_i$

In a calcium-containing external solution, the mean basal $[Ca^{2+}]_i$ level in A7r5 was 82.7 ± 4.3 nM ($n = 66$), and was unchanged throughout the experimental time course. Cumulative addition of *c*-RESV or *t*-RESV (0.1 – 100 μM) caused a concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 2), which did not return to the resting level within 1 h, even after washing out the drugs. Maximal $[Ca^{2+}]_i$ levels reached were 246.7 ± 35.6 nM, $n = 14$ and 270.0 ± 27.9 nM, $n = 17$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them. $EC_{50}$: 16.5 ± 4.4 μM and 6.8 ± 1.3 μM, for *t*-RESV and *c*-RESV, respectively; $p < 0.05$. The

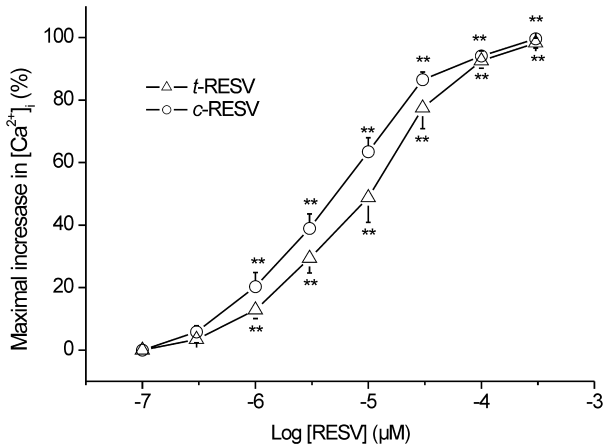


**Figure 2.** Cumulative concentration-response curves for *t*-RESV- or *c*-RESV-induced increases in $[Ca^{2+}]_i$ in a 1.5 mM $Ca^{2+}$-containing external solution in A7r5 cells. Responses are expressed as % of the maximal response obtained for each compound, considering the basal values as 0%. Each point represents the mean of 14 or 17 experiments for *t*-RESV and *c*-RESV, respectively, with SEM indicated by vertical bars. Statistical significance with respect to basal values: $^{**}p < 0.01$.

percentage of cells responding to *c*-RESV and *t*-RESV in a given culture did not vary greatly, and was on average ~90% of the cells that responded to 0.5 μM ionomycin at the end of the experiments.

 

### 3.2 Effects of $Ni^{2+}$, nifedipine, and SKF 96365 on RESV-induced rises in $[Ca^{2+}]_i$

A single application of *t*-RESV or *c*-RESV (10 μM) caused a sustained increase in $[Ca^{2+}]_i$ (maximal $[Ca^{2+}]_i$ levels: 201.9 ± 41.3 nM, $n = 19$ and 212.6 ± 39.8 nM, $n = 16$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them). Application of $Ni^{2+}$ (300 μM) significantly inhibited the $[Ca^{2+}]_i$-sustained plateau when it was administered 20 min after the application of *t*-RESV or *c*-RESV (reduction of the maximal response to both isomers of RESV: ~50%; Fig. 3a). Similarly, application of nifedipine (1 μM) partially reduced the *t*-RESV- or *c*-RESV-induced increase in $[Ca^{2+}]_i$ (reduction of the maximal response to both isomers of RESV: ~20%; Fig. 3a). However, SKF 96365 (30 μM) did not significantly modify the *t*-RESV- or *c*-RESV-induced $[Ca^{2+}]_i$-sustained plateau (Fig. 3a).

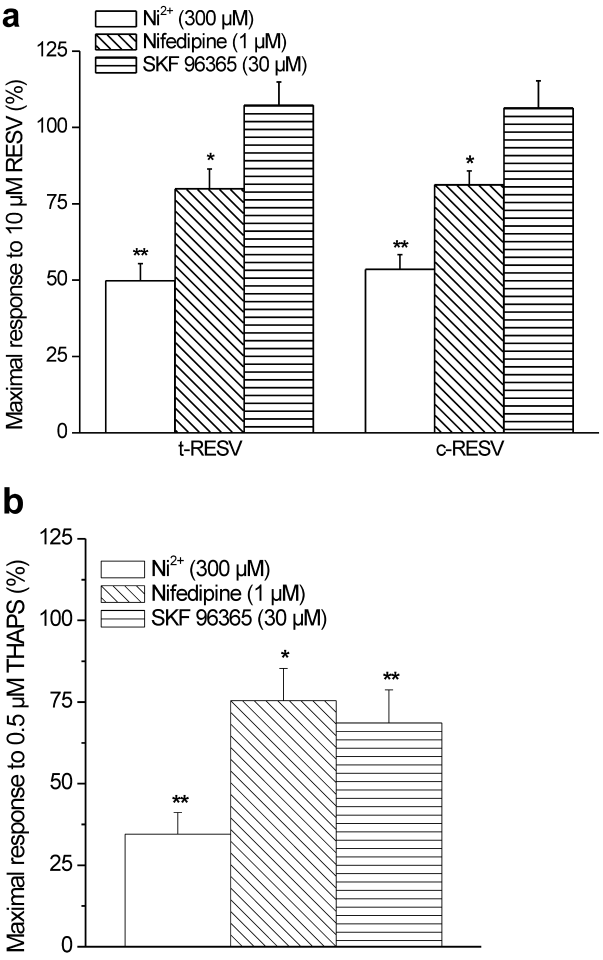


**Figure 3.** Effects of $Ni^{2+}$, nifedipine or SKF 96365 on the maximal increase in $[Ca^{2+}]_i$ (considered as the 100%) induced by (a) RESV or (b) THAPS in a 1.5 mM $Ca^{2+}$-containing external solution. Each bar represents the mean ± SEM (indicated by vertical lines) of at least 12 cells. * $p < 0.05$ or ** $p < 0.01$ *versus* control values.

### 3.3 Interferences between THAPS- and RESV-induced increases in $[Ca^{2+}]_i$

#### 3.3.1 Effects of previous administration of THAPS on RESV-induced increases in $[Ca^{2+}]_i$

In a 1.5 mM $CaCl_2$-containing bathing solution, previous incubation with THAPS affects the *t*-RESV- and *c*-RESV-induced increase in $[Ca^{2+}]_i$ depending on the concentration used (Table 1). THAPS (10 nM) did not significantly modify basal $[Ca^{2+}]_i$ levels ($[Ca^{2+}]_i$ ~ 5 min after the application of THAPS: 82.0 ± 8.1 nM, $n = 10$; $p > 0.05$) and THAPS (100 nM) induced a slow transient increase of $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$ 5 min after the application of THAPS: 159.0 ± 15.4 nM, $n = 14$; $p < 0.01$). 10 min after the application of THAPS (10, 100 nM), *t*-RESV or *c*-RESV (10 μM) induced an increase in $[Ca^{2+}]_i$ that was significantly higher than in the absence of THAPS (Table 1). The subsequent application of a higher concentration of both isomers of RESV (30 μM) induced an additional increase in $[Ca^{2+}]_i$ in the presence of 10 nM THAPS but not in the presence of 100 mM THAPS (Table 1).

Higher concentrations of THAPS induced an increase in $[Ca^{2+}]_i$, consisting of an initial peak after 3–5 min ($[Ca^{2+}]_i$ ~5 min after the application of 0.5 or 1 μM THAPS: 332.6 ± 34.3 nM, $n = 16$ and 361.3 ± 36.9 nM, $n = 14$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them) that decreased to a sustained plateau ($[Ca^{2+}]_i$ ~ 15 min after the application of 0.5 or 1 μM THAPS: 143.1 ± 13.3 nM, $n = 16$ and 168.2 ± 16.8 nM, $n = 14$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them). The THAPS-induced plateau was significantly inhibited by $Ni^{2+}$ (300 μM), nifedipine (1 μM) or SKF96365 (30 μM) (reduction of the maximal response to 1 μM THAPS: ~65%; ~25% and ~30%, respectively; Fig. 3b). The application of *t*-RESV or *c*-RESV (10, 30 μM) on the sustained plateau induced by THAPS (0.5, 1 μM) induced an additional increase in $[Ca^{2+}]_i$ which was not significantly higher, or even significantly reduced, compared to the values obtained in absence of THAPS (Table 1).

The additive increase in $[Ca^{2+}]_i$ induced by the application of THAPS and *t*-RESV or *c*-RESV was inhibited in part by $Ni^{2+}$ (300 μM; ~60% reduction) and nifedipine (1 μM, ~20% reduction), but not by SKF 96365 (30 μM) (data not shown).

#### 3.3.2 Effects of previous administration of RESV on THAPS-induced increases in $[Ca^{2+}]_i$

In a 1.5 mM $CaCl_2$-containing bathing solution, THAPS-induced rises in $[Ca^{2+}]_i$ were significantly affected by a previous administration of RESV. Lower concentrations of *t*-RESV or *c*-RESV (100 nM) did not induce by themselves significant increases in $[Ca^{2+}]_i$. Under these conditions, the increase in $[Ca^{2+}]_i$ induced by the subsequent administration

 

**Table 1.** Effects of the previous administration of THAPS on the *t*-RESV- and *c*-RESV-induced increases in $[Ca^{2+}]_i$ (nM) in a 1.5 mM $Ca^{2+}$-containing external solution in A7r5 cells

| | | *t*-RESV | | *c*-RESV | |
|---|---|---|---|---|---|
| | | 10 μM | 30 μM | 10 μM | 30 μM |
| Control | | 201.9 ± 41.3 | 258.9 ± 33.1 | 212.6 ± 39.8 | 245.0 ± 26.6 |
| | 10 nM | 299.2 ± 22.2** | 336.7 ± 32.0** | 278.3 ± 37.7* | 327.4 ± 22.3** |
| Previous | 100 nM | 343.4 ± 31.1** | 345.2 ± 44.9** | 326.8 ± 26.4** | 319.1 ± 25.8** |
| THAPS | 500 nM | 220.3 ± 30.1 | 208.2 ± 19.0* | 229.3 ± 19.1 | 216.32 ± 21.52 |
| | 1 μM | 189.2 ± 16.2 | 178.2 ± 15.6** | 201.5 ± 24.8 | 177.2 ± 21.7** |

Each value is mean ± SEM of at least 12 cells. Level of statistical significance: * $p < 0.05$ or ** $p < 0.01$ with respect to control values.

**Table 2.** Effects of the previous administration of *t*-RESV- and *c*-RESV on the THAPS-induced increases in $[Ca^{2+}]_i$ (nM) in a 1.5 mM $Ca^{2+}$-containing external solution in A7r5 cells

| | | THAPS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 10 nM | | 100 nM | | 500 nM | | 1 μM | |
| | | Peak | Plateau | Peak | Plateau | Peak | Plateau | Peak | Plateau |
| Control | | 82.0 ± 8.1 | 78.3 ± 5.5 | 159.0 ± 15.4 | 90.6 ± 7.4 | 332.6 ± 34.3 | 143.1 ± 13.3 | 361.3 ± 36.9 | 168.2 ± 16.7 |
| Previous | 100 nM | 129.8 ± 21.2** | 85.9 ± 7.1 | 208.1 ± 31.1** | 116.4 ± 15.0* | 395.0 ± 24.4* | 177.3 ± 19.0 ** | 377.9 ± 28.9 | 184.3 ± 23.4 |
| *t*-RESV | 10 μM | 198.4 ± 30.0** | 135.3 ± 16.9** | 215.2 ± 28.9** | 142.2 ± 28.1** | 256.3 ± 22.0** | 122.1 ± 19.5 | 235.4 ± 33.7** | 119.8 ± 15.1** |
| Previous | 100 nM | 135.2 ± 17.2** | 90.6 ± 10.0 | 210.8 ± 19.1** | 122.4 ± 27.0 | 401.9 ± 29.3** | 190.2 ± 22.6** | 398.1 ± 30.0 | 192.3 ± 24.5 |
| *c*-RESV | 10 μM | 205.4 ± 21.6** | 140.0 ± 17.7** | 233.8 ± 31.5** | 139.8 ± 18.4** | 277.2 ± 27.8** | 130.1 ± 17.5 | 244.4 ± 31.1** | 122.3 ± 23.3** |

Each value is mean ± SEM of at least 10 cells. Level of statistical significance: * $p < 0.05$ or ** $p < 0.01$ with respect to control values.

of THAPS (10, 100, 500 nM) was significantly potentiated (Table 2). However, the response to the higher concentration of THAPS (1 μM) was not significantly modified (Table 2). A higher concentration of *t*-RESV or *c*-RESV (10 μM), which induced a marked increase in $[Ca^{2+}]_i$ (see above), did significantly potentiate the increase in $[Ca^{2+}]_i$ induced by THAPS (10, 100 nM), but did reduce the increase in $[Ca^{2+}]_i$ induced by THAPS (0.5, 1 μM) (Table 2).

### 3.4 Effects of *t*-RESV and *c*-RESV on basal $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$

In a free-calcium external solution, the average basal $[Ca^{2+}]_i$ was 65.7 ± 5.7 nM ($n = 33$; $p < 0.05$ with respect to that obtained in 1.5 mM $Ca^{2+}$ external solution), and was unchanged throughout the experimental time course. Addition of *c*-RESV or *t*-RESV (10 μM) induced a transient increase in $[Ca^{2+}]_i$ that reaches its maximum after 8–10 min and returns to its basal levels after ~25 min. The maximal $[Ca^{2+}]_i$ level reached were 115.6 ± 12.5 nM, $n = 18$ and 111.2 ± 13.0 nM, $n = 15$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them. Repetitive and cumulative addition of both RESV isomers was able to induce new increases in $[Ca^{2+}]_i$ although the maximal level continued to be reduced until the complete absence of response after 3–4 applications.

After a single application of *t*-RESV or *c*-RESV (10 μM), the replacement of the free-$Ca^{2+}$ solution by a calcium-containing one (1.5 mM $CaCl_2$), induced an increase in $[Ca^{2+}]_i$ following the model of the classic capacitative entry pathway [18]. The maximal $[Ca^{2+}]_i$ levels reached were 163.1 ± 18.3 nM, $n = 9$ and 158.4 ± 26.1 nM, $n = 7$, respectively; $p < 0.01$ with respect to basal values; $p > 0.05$ between them). *t*-RESV or *c*-RESV (100 μM) induced an increase in $[Ca^{2+}]_i$ that was significantly higher than that induced by lower concentrations (10 μM) of these agents. Under these conditions cells did not respond to a second application of the drugs (data not shown).

### 3.5 Interferences between THAPS- and RESV-induced rises in $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$

In a free-calcium external solution, the addition of THAPS (0.5 μM) induced a transient increase in $[Ca^{2+}]_i$ that reached its maximum after 3–5 min (maximal $[Ca^{2+}]_i$ level: 184.3 ± 20.9 nm, $n = 12$; $p < 0.01$ with respect to basal values) and returned to its basal levels after ~10 min. After the application of THAPS, the replacement of the free-$Ca^{2+}$ solution by a calcium-containing one (1.5 mM $CaCl_2$), induced a capacitative $Ca^{2+}$ entry (maximal $[Ca^{2+}]_i$: 163.1 ± 25.1 nM, $n = 10$; $p < 0.01$ with respect to basal values).

 

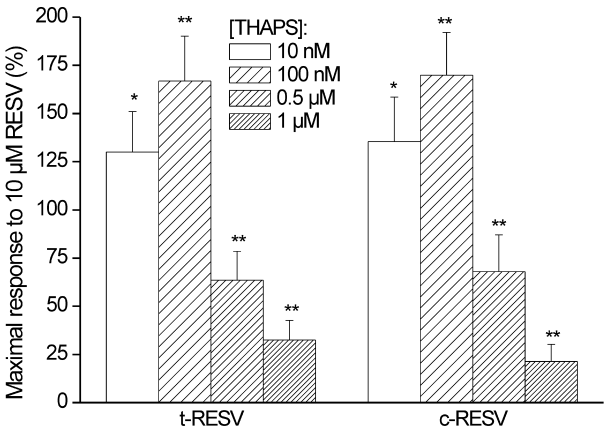


**Figure 4.** Effects of previous application of THAPS on the maximal increase in $[Ca^{2+}]_i$ (considered as the 100%) induced by *t*-RESV or *c*-RESV in the absence of extracellular $Ca^{2+}$. Each bar represents the mean ± SEM (indicated by vertical lines) of at least 10 cells. * $p < 0.05$ or ** $p < 0.01$ *versus* control values.

The interactions between THAPS and both isomers of RESV correlates well with the results obtained in a 1.5 mM $CaCl_2$-containing bath solution. Thus, the RESV-induced increases in $[Ca^{2+}]_i$ were potentiated by a previous application of low concentrations of THAPS (10, 100 nM). However, higher concentrations of THAPS (0.5, 1 µM) did significantly inhibit the *t*-RESV- or *c*-RESV-induced increase in $[Ca^{2+}]_i$ (Fig. 4).

Furthermore, after partial depletion of intracellular $Ca^{2+}$ stores with *t*-RESV or *c*-RESV (10 µM), THAPS-induced (0.5 µM) increases in $[Ca^{2+}]_i$, were significantly reduced (maximal $[Ca^{2+}]_i$: 111.9 ± 12.5 nM, $n = 11$ and 98.0 ± 14.2 nM, $n = 13$ after *t*-RESV and *c*-RESV, respectively; $p < 0.01$ with respect to values in the absence of RESV). Under the conditions when *t*-RESV or *c*-RESV were not able to release more intracellular $Ca^{2+}$ (*i.e.*, after application of 100 µM *t*-RESV or *c*-RESV of after cumulative applications of 10 µM *t*-RESV or *c*-RESV), subsequent application of THAPS (0.5 µM) did not increase basal $[Ca^{2+}]_i$ levels.

### 3.6 Effects of TMB-8 on *t*-RESV-, *c*-RESV-, or THAPS-induced rises in $[Ca^{2+}]_i$

In the absence of extracellular $Ca^{2+}$, 5 min of preincubation with the intracellular $Ca^{2+}$ antagonist TMB-8 (100 µM) did completely inhibit the $[Ca^{2+}]_i$ increases induced by THAPS (0.5 µM) or by both isomers of RESV (10 µM). However, after 5 min of preincubation with TMB-8 (100 µM) in the presence of 1.5 mM external $Ca^{2+}$, THAPS (0.5 µM), *t*-RESV (10 µM), or *c*-RESV (10 µM) were still able to induce a slow, sustained increase in $[Ca^{2+}]_i$ (maximal $[Ca^{2+}]_i$ levels: 142.5 ± 16.0 nM, $n = 9$, 129.1 ± 15.1 nM, $n = 12$ and 119.7 ± 18.3 nM, $n = 8$, respectively; $p < 0.01$ with respect to basal values), suggesting that these agents can induce a transmembrane $Ca^{2+}$ influx by other mechanism than the activation of store-operated $Ca^{2+}$ channels (SOCCs). These increases in $[Ca^{2+}]_i$ were not significantly inhibited by the application of $Ni^{2+}$ (300 µM), nifedipine (1 µM), or SKF 96365 (30 µM).

## 4 Discussion

This is the first comparative study of the potential effects of both isomers of RESV on $[Ca^{2+}]_i$ in A7r5 cells. In our experiments, the pharmacological effects caused by *t*-RESV and *c*-RESV were qualitatively similar, *i.e.*, they directly increased $[Ca^{2+}]_i$, which suggests that the different spatial conformation of *c*-RESV (*versus* that of the *trans*-isomer) does not seem to modify markedly its interaction with the potential cellular targets in A7r5 cells. In this connection, it is interesting to note that, according to their $EC_{50}$ values, *c*-RESV was slightly, but significantly more effective than *t*-RESV.

Since $[Ca^{2+}]_i$ variations directly regulate vascular contraction, it would be reasonable to speculate that the RESV-induced increase of $[Ca^{2+}]_i$ levels in A7r5 is correlated to an augmentation of the resting tone of isolated rat aorta. However, previous reports have shown that *t*-RESV directly relaxes rat aortic smooth muscle [8, 9] (see Section 1). These discrepancies could be due to the following reasons:
i) A different sensitivity to RESV of the A7r5 cloned cell line compared to the rat aortic smooth muscle cells (RASMCs) isolated from the medial layer of rat aorta (for detailed method of preparation, see [19]). In order to exclude or confirm this possibility, further investigations are currently being conducted in our laboratory to establish whether RESV also induces increases in $[Ca^{2+}]_i$ in RASMCs.
ii) The increase in $[Ca^{2+}]_i$ measured by fura-2 imaging techniques in isolated RASMCs is not always accompanied by an augmentation of the basal tone in rat aortic rings. In fact, THAPS-induced transient rise in $[Ca^{2+}]_i$ in RASMC, in the absence of extracellular $Ca^{2+}$, is not accompanied by a contraction in rat aorta rings [20], suggesting that intracellular $Ca^{2+}$ release by this drug in RASMCs is not coupled to muscular contraction. However, controversial results have been reported on the relationship between $[Ca^{2+}]_i$ elevation after inhibition of the sarcoplasmic reticulum $Ca^{2+}$-ATPase and contraction in vascular smooth muscle [20–22].
iii) The RESV-induced increase in $[Ca^{2+}]_i$ in A7r5 cells may be counteracted in the myocytes of isolated rat aortic preparations by other mechanisms contributing to the vasorelaxant effects of RESV, *i.e.*, the inhibitory effects of RESV

 

on NADH/NADPH oxidase enzymatic activity [5] or the capacity to inhibit some of the steps of the contractile effect induced by PKC [9] (see also Section 1). In this regard, the possibility of RESV acting as an inhibitor of agonist-induced increases in $[Ca^{2+}]_i$ by another mechanism (*i. e.*, a blockade of nonselective cation channels) can not be discarded from our experiments. In fact, in the present work, both *t*-RESV and *c*-RESV did significantly reduce the increases in $[Ca^{2+}]_i$ induced by the higher concentrations of THAPS (0.5 – 1 μM). Furthermore, in another set of experiments performed in our laboratory, *t*-RESV did significantly decrease the plateau of the response to vasopressin, before inducing a slow increase in $[Ca^{2+}]_i$ in A7r5 cells (unpublished observations). Finally, after partial depletion of intracellular $Ca^{2+}$ stores by RESV, the response to a subsequent application of an agonist acting through a phospholipase C and inositol 1,4,5-triphosphate-mediated mechanism, could be significantly reduced. Supporting this hypothesis, pre-incubation with low concentrations of *t*-RESV significantly reduces the vasopressin-induced increase in $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$ in A7r5 cells (unpublished observations). Thus, it seems that the effects of RESV isomers on $[Ca^{2+}]_i$ in A7r5 cells depend on the state of refilling of the intracellular stores and, under some conditions, an inhibitory effect may be more important than the $[Ca^{2+}]_i$-increasing effect described here. Further studies are needed to confirm this possibility.

In our experiments in free-$Ca^{2+}$ external solution, both *t*-RESV and *c*-RESV induced an increase in $[Ca^{2+}]_i$ as a consequence of the depletion of intracellular stores. TMB-8, an inhibitor of intracellular $Ca^{2+}$ release that reduces $Ca^{2+}$ availability in smooth and skeletal muscles by stabilizing $Ca^{2+}$ binding to cellular $Ca^{2+}$ stores [23], has been shown to transiently block THAPS-induced rise in $[Ca^{2+}]_i$ for about 20 min [24] and to abolish the initial peak of the contractile response induced by THAPS in rat aortic strips [25]. In the same work, Tepel *et al.* [25] have also shown that the second phase of the response to THAPS was not inhibited by TMB-8, but it was completely abolished in the absence of extracellular $Ca^{2+}$. According to this, in our experiments in the absence of extracellular $Ca^{2+}$, TMB-8 completely abolished the increase in $[Ca^{2+}]_i$ induced by THAPS. A similar effect of TMB-8 was observed on *t*-RESV- or *c*-RESV-induced increases in $[Ca^{2+}]_i$, suggesting than both stilbenes, as well as THAPS, release $Ca^{2+}$ from intracellular stores which are sensitive to the action of TMB-8.

The interferences between THAPS- and RESV-induced responses in the absence of extracellular $Ca^{2+}$ indicate that these drugs are probably acting on the same intracellular stores. Thus, after partial depletion of THAPS-sensitive intracellular stores, the *t*-RESV- and *c*-RESV-induced increases in $[Ca^{2+}]_i$ were significantly reduced. However, after applications of lower concentrations of THAPS (which did not induce an increase of $[Ca^{2+}]_i$ by themselves), the response to both isomers of RESV was significantly potentiated. A similar situation was produced when the RESV isomers were administered before THAPS. The possibility of RESV inhibiting sarcoplasmic reticulum $Ca^{2+}$-ATPase in a similar way that THAPS can not be ruled out, since an inhibition of ATPase activity has already been described for *t*-RESV [26, 27].

It has been described that depletion of intracellular $Ca^{2+}$ pools, either by membrane agonists or by intracellular agents, increases membrane permeability to $Ca^{2+}$ in non-excitable cells, which, in turn, permits the refilling of depleted stores through SOCCs, in accordance with the so-called "capacitative model" [18, 28]. This $Ca^{2+}$ influx can be inhibited with SKF 96365 [29]. However, the involvement of other channels, such as dihydropyridine-sensitive $Ca^{2+}$ channels, is not clear, and contradictory results have been reported [30, 31].

Here, the release of $Ca^{2+}$ from intracellular stores induced by THAPS, *t*-RESV, or *c*-RESV was followed by a transmembrane capacitative $Ca^{2+}$ influx, since reintroduction of external $Ca^{2+}$ in THAPS-, *t*-RESV-, or *c*-RESV-depleted cells elicited a sustained increase in $[Ca^{2+}]_i$. The RESV- and the THAPS-induced influx of $Ca^{2+}$ through SOOCs was partially inhibited by $Ni^{2+}$, an inhibitor of capacitative $Ca^{2+}$ entry or nifedipine, a voltage-dependent L-type $Ca^{2+}$ channel blocker. However, SKF 96365, a nonselective $Ca^{2+}$ channel inhibitor that also reduces $Ca^{2+}$ influx through SOCC (see above), only inhibited the increase in $[Ca^{2+}]_i$ induced by THAPS, but not by *t*-RESV or *c*-RESV. This lack of effects of SKF 96365 could be explained on the basis that, under some conditions, this imidazole has been reported to increase intracellular $[Ca^{2+}]_i$ by releasing $Ca^{2+}$ from intracellular stores in A7r5 cells [32]. Then, the inhibitory effect of SKF 96365 on SOCCs could be more evident on THAPS-induced capacitative $Ca^{2+}$ influx, which was significantly higher than that induced by the RESV isomers.

Mechanisms other than the activation of SOCCs could also be implicated in the increase in $[Ca^{2+}]_i$ induced by both isomers of RESV, since in the presence of 1.5 mM extracellular $Ca^{2+}$ TMB-8 was not able to completely inhibit the rise in $[Ca^{2+}]_i$, suggesting that, when the release of $Ca^{2+}$ from intracellular stores was blocked, *t*-RESV or *c*-RESV were still able to induce a transmembrane $Ca^{2+}$ influx, which was not significantly affected by $Ni^{2+}$, nifedipine or SKF 96365. A similar result was obtained for THAPS, in good agreement with the results of Tepel *et al.* [25] (see above).

In conclusion, we have described for the first time that *t*-RESV and *c*-RESV, either acting at the plasma membrane and/or by direct diffusion or active transport into the cytoplasm, induce an unexpected increase in $[Ca^{2+}]_i$ the *cis*-isomer being slightly but significantly more effective than the

 

*trans*-isomer. This increase in $[Ca^{2+}]_i$ is mediated by depletion of $Ca^{2+}$ from the same intracellular stores released by thapsigargin, and subsequent influx of $Ca^{2+}$ through $Ni^{2+}$- and nifedipine-sensitive SOCCs, although a direct activation of transmembrane $Ca^{2+}$ influx through another type of channel that were not affected by $Ni^{2+}$, nifedipine, or by SKF 96365 may be also implicated.

Taking into account the previous reports on the effects of *t*-RESV in rat aortic preparations (see Section 1), the RESV-induced increase in $Ca^{2+}$ seems to be not relevant for inducing contraction of rat aortic smooth muscle. However, a similar increase in $[Ca^{2+}]_i$ in endothelial cells could induce an increase of the NO release, and thus contribute to the endothelium-dependent vasorelaxant effect reported for *t*-RESV in rat aorta [5, 8]. In fact, substances that increase endothelial cell $[Ca^{2+}]_i$ potentially influence the production of endothelial factors (notably NO, because of the $Ca^{2+}$ sensitivity of constitutive NO synthase in the endothelium) [33] and underlying vascular tone, as reported for the ionophore A23187 and for THAPS, which induce endothelium-dependent relaxation of Guinea-pig rat aorta [34, 35].

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## 5 References

[1] Soleas, G. J., Diamandis, E. P., Goldberg, D. M., The world of resveratrol. *Adv. Exp. Med. Biol.* 2001, *492*, 159 – 182.

[2] Pervaiz, S., Resveratrol: from grapevines to mammalian biology. *FASEB J.* 2003, *17*, 1975 – 1985.

[3] Fremont, L., Biological effects of resveratrol. *Life Sci.* 2000, *66*, 663 – 673.

[4] Aggarwal, B. B., Bhardwaj, A., Aggarwal, R. S., Seeram, N. P., *et al.*, Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res.* 2004, *24*, 2783 – 2840.

[5] Orallo, F., Álvarez, E., Camiña, M., Leiro, J. M., *et al.*, The possible implication of *trans*-resveratrol in the cardioprotective effects of long-term moderate wine consumption. *Mol. Pharmacol.* 2002, *61*, 294 – 302.

[6] Bradamante, S., Barenghi, L., Villa, A., Cardiovascular protective effects of resveratrol. *Cardiovasc. Drug Rev.* 2004, *22*, 169 – 188.

[7] Fitzpatrick, D. F., Hirschfield, S. L., Coffey, R. G., Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am. J. Physiol.* 1993, *265*, H774 – H778.

[8] Chen, C. K., Pace-Asciak, C. R., Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. *Gen. Pharmacol.* 1996, *27*, 363 – 366.

[9] Orallo, F., Camiña, M., Study of the endothelium-dependent and endothelium-independent vasodilator effects of resveratrol in rat aorta. *Br. J. Pharmacol.* 1998, *124*, 108P.

[10] Leiro, J., Álvarez, E., Arranz, J. A., Laguna, R., *et al.*, Effects of *cis*-resveratrol on inflammatory murine macrophages: antioxidant activity and down-regulation of inflammatory genes. *J. Leukoc. Biol.* 2004, *75*, 1156 – 1165.

[11] Bertelli, A. A., Giovannini, L., Bernini, W., Migliori, M., *et al.*, Antiplatelet activity of *cis*-resveratrol. *Drugs Exp. Clin. Res.* 1996, *22*, 61 – 63.

[12] Basly, J. P., Marre-Fournier, F., Le Bail, J. C., Habrioux, G., Chulia, A. J., Estrogenic/antiestrogenic and scavenging properties of (E)- and (Z)-resveratrol. *Life Sci.* 2000, *66*, 769 – 777.

[13] Waffo-Teguo, P., Hawthorne, M. E., Cuendet, M., Merillon, J. M., *et al.*, Potential cancer-chemopreventive activities of wine stilbenoids and flavans extracted from grape (*Vitis vinifera*) cell cultures. *Nutr. Cancer* 2001, *40*, 173 – 179.

[14] Rotondo, S., Rajtar, G., Manarini, S., Celardo, A., *et al.*, Effect of *trans*-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leukocyte function. *Br. J. Pharmacol.* 1998, *123*, 1691 – 1699.

[15] Dobrydneva, Y., Williams, R. L., Blackmore, P. F., *trans*-Resveratrol inhibits calcium influx in thrombin-stimulated human platelets. *Br. J. Pharmacol.* 1999, *128*, 149 – 157.

[16] Dobrydneva, Y., Williams, R. L., Morris, G. Z., Blackmore, P. F., Dietary phytoestrogens and their synthetic structural analogues as calcium channel blockers in human platelets. *J. Cardiovasc. Pharmacol.* 2002, *40*, 399 – 410.

[17] Grynkiewicz, G., Poenie, M., Tsien, R. Y., A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 1985, *260*, 3440 – 3450.

[18] Putney, J. W., Jr., Capacitative calcium entry revisited. *Cell Calcium* 1990, *11*, 611 – 624.

[19] Orallo, F., Study of the *in vivo* and *in vitro* cardiovascular effects of a hydralazine-like vasodilator agent (HPS-10) in normotensive rats. *Br. J. Pharmacol.* 1997, *121*, 1627 – 1636.

[20] Álvarez-Castro, E., Campos-Toimil, M., Orallo, F., (–)-Epigallocatechin-3-gallate induces contraction of the rat aorta by a calcium influx-dependent mechanism. *Naunyn Schmiedeberg's Arch. Pharmacol.* 2004, *369*, 496 – 506.

[21] Tosun, M., Paul, R. J., Rapoport, R. M., Coupling of store-operated $Ca^{++}$ entry to contraction in rat aorta. *J. Pharmacol. Exp. Ther.* 1998, *285*, 759 – 766.

[22] Zhang, X. F., Iwamuro, Y., Enoki, T., Okazawa, M., *et al.*, Pharmacological characterization of $Ca^{2+}$ entry channels in endothelin-1- induced contraction of rat aorta using LOE 908 and SK&F 96365. *Br. J. Pharmacol.* 1999, *127*, 1388 – 1398.

[23] Chiou, C. Y., Malagodi, M. H., Studies on the mechanism of action of a new $Ca^{2+}$-antagonist, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. *Br. J. Pharmacol.* 1975, *53*, 279 – 285.

[24] Doutheil, J., Paschen, W., Effect of 3,4,5-trimethoxybenzoic acid 8-diethylamino-octyl ester (TMB-8) on neuronal calcium homeostasis, protein synthesis, and energy metabolism. *Pharmacol. Toxicol.* 1999, *85*, 85 – 91.

 

[25] Tepel, M., Ruess, C., Mehring, N., Neusser, M., Zidek, W., Effect of inhibition of sarcoplasmic Ca(2+)-ATPase on vasoconstriction and cytosolic $Ca^{2+}$ in aortic smooth muscle from spontaneously hypertensive and normotensive rats. *Clin. Exp. Hypertens.* 1994, *16*, 493 – 506.

[26] Zini, R., Morin, C., Bertelli, A., Bertelli, A. A., Tillement, J. P., Effects of resveratrol on the rat brain respiratory chain. *Drugs Exp. Clin. Res.* 1999, *25*, 87 – 97.

[27] Arakaki, N., Nagao, T., Niki, R., Toyofuku, A., *et al.*, Possible role of cell surface $H^+$-ATP synthase in the extracellular ATP synthesis and proliferation of human umbilical vein endothelial cells. *Mol. Cancer Res.* 2003, *1*, 931 – 939.

[28] Randriamampita, C., Tsien, R. Y., Emptying of intracellular $Ca^{2+}$ stores releases a novel small messenger that stimulates $Ca^{2+}$ influx. *Nature* 1993, *364*, 809 – 814.

[29] Miwa, S., Iwamuro, Y., Zhang, X. F., Inoki, T., *et al.*, $Ca^{2+}$ entry channels in rat thoracic aortic smooth muscle cells activated by endothelin-1. *Jpn. J. Pharmacol.* 1999, *80*, 281 – 288.

[30] Samain, E., Bouillier, H., Miserey, S., Perret, C., *et al.*, Extracellular signal-regulated kinase pathway is involved in basic fibroblast growth factor effect on angiotensin II-induced $Ca^{(2+)}$ transient in vascular smooth muscle cell from Wistar-Kyoto and spontaneously hypertensive rats. *Hypertension* 2000, *35*, 61 – 67.

[31] Purdy, K. E., Arendshorst, W. J., Iloprost inhibits inositol-1,4,5-trisphosphate-mediated calcium mobilization stimulated by angiotensin II in cultured preglomerular vascular smooth muscle cells. *J. Am. Soc. Nephrol.* 2001, *12*, 19 – 28.

[32] Hughes, A. D., Schachter, M., Multiple pathways for entry of calcium and other divalent cations in a vascular smooth muscle cell line (A7r5). *Cell Calcium* 1994, *15*, 317 – 330.

[33] Hobbs, A. J., Higgs, A., Moncada, S., Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu. Rev. Pharmacol. Toxicol.* 1999, *39*, 191 – 220.

[34] Taniguchi, H., Hirano, H., Tanaka, Y., Tanaka, H., Shigenobu, K., Possible involvement of $Ca^{2+}$ entry and its pharmacological characteristics responsible for endothelium-dependent, NO-mediated relaxation induced by thapsigargin in Guinea-pig aorta. *J. Pharm. Pharmacol.* 1999, *51*, 831 – 840.

[35] Taniguchi, H., Tanaka, Y., Hirano, H., Tanaka, H., Shigenobu, K., Evidence for a contribution of store-operated $Ca^{2+}$ channels to NO-mediated endothelium-dependent relaxation of Guinea-pig aorta in response to a $Ca^{2+}$ ionophore, A23187. *Naunyn Schmiedeberg's Arch. Pharmacol.* 1999, *360*, 69 – 79.

 