Journal of Animal &Plant Sciences, 2013. Vol.17, Issue 2: 2540-2551
Publication date 9/4/2013, http://www.m.elewa.org/JAPS*; ISSN 2071-7024*

Intracellular Ca^{2+} signalling concentration increase induced by 3β-16β, 23, 29-tetrahydroxyoleane-12-ene (THO) on rat aorta endothelial cells.

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Keywords: 3β-16β, 23, 29-tetrahydroxyoleane-12-ene, aorta, Ca^{2+} signalling, endothelial Cells

1 SUMMARY

Ca^{2+} signalling induced by 3β-16β, 23, 29-tetrahydroxyoleane-12-ene (10^{-4} M) in *in situ* endothelium of aortic rings was evaluated to investigated the mechanisms underlying intracellular Ca^{2+} signalling in rat aortic endothelium loaded with the Ca^{2+}-sensitive dye, fura-2/AM. In situ endothelium cells were visualized by an upright epifluorescence Axiolab microscope. Cytoplasm-free calcium concentration $[Ca^{2+}]$ i was estimated by determining the fluorescence ratio of the $[Ca^{2+}]$ i probes, Fura 2/AM. This study showed that THO caused a slow, long lasting increase in the $[Ca^{2+}]_i$ of aortic endothelial cells. Such a slow $[Ca^{2+}]_i$ increase was very limited in Ca^{2+}-free extracellular medium. THO, administrated in Ca^{2+} free medium did not increase intracellular influx Ca^{2+}. What this suggests is that THO would act on the calcium channels by stimulating their openings, thus allowing a massive entry of Ca^{2+} into the cell. The lack of Ca^{2+} entry was verified by the failure of ATP to produce increase amplitude in the aortic rings in the absence of extracellular Ca^{2+}. The study showed that the slow increase in $[Ca^{2+}]_i$ did not involve the sarcoplasmic reticulum Ca^{2+}-pump, through pre-treatment of our preparations with Cyclopiazonic acid (10 mM) inhibitor of sarcoplasmic reticulum Ca^{2+}-pump. To determine if THO-induced intracellular influx Ca^{2+} involves the participation of channels calcium, the preparations were pre-treated with Lanthanum III the non-specific calcium channel antagonists and the result showed that lanthanum III (100 µM) completely abolished the higher amplitude due to THO effect. THO-induced intracellular influx Ca^{2+} involves the participation of channels calcium.

Abbreviations:
THO: 3β-16β, 23, 29-tetrahydroxyoleane-12-ene; NO: nitric oxide; La^{3+}: Lanthanum III; ATP: Adenosine triphosphste; CPA: Cyclopiazonic acid; $0Ca^{2+}$: absence of extracellular Ca^{2+}; $[Ca^{2+}]_i$: intracellular Ca^{2+} concentration; PSS: physiological salt solution; ECs: Endothelium cells, IP_3: inositol 1, 4, 5-trisphosphate

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2 INTRODUCTION

In endothelial cells (ECs), an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the triggering event in the synthesis and release of a number of vasoactive compounds, such as prostacyclin, nitric oxide and endothelins (Himmel *et al*, 1993, Inagami *et al*, 1995). Several local mediators (histamine, ATP, and bradykinin) are able to promote $[Ca^{2+}]_i$ increases by elevating the intracellular level of inositol 1,4,5-trisphosphate (IP_3), thus causing Ca^{2+} release from endoplasmic-reticulum Ca^{2+} stores through IP_3-sensitive channels (Berridge 1993). Phytochemical investigation of the bark of trunk of *Vepris heterophylla* (Engl.) R. Let. (Rutaceae), a medicinal plant used empirically in Cameroon by traditional healers in the treatment of various illnesses such as cardio-vascular disorders, especially arterial hypertension, and renal disorders, led to the isolation of two major components among which the triterpene, 3β-16β,23,29-tetrahydroxyoleane-12-ene (THO). The concentration of intracellular calcium controls many fundamental events such as muscle contraction, vasorelaxation, neurotransmitter synthesis or release. **(**Berridge *et al.*, 1998). Particularly, in the aorta ECs, the production of the endothelium-derived relaxing factors is triggered by an increase of calcium concentration in the cytoplasm of the ECs, and the tonus of smooth muscles is controlled by their intracellular calcium concentration (Chen and Suzuki, 1990; Förstermann *et al.*, 1991; Somlyo and Somlyo, 1994). The plasma membrane Ca^{2+} ATPase (PMCA) is a transport protein in the plasma membrane of cells that serves to remove Ca^{2+} from the cell. It is vital for regulating the amount of Ca^{2+} within cells (Strehler *et al.*, 2001). There is a very large transmembrane electrochemical gradient of Ca^{2+} driving the entry of the ion into cells , yet it is very important for cells to maintain low concentrations of Ca^{2+} for proper cells signalling, thus it is necessary for the cells to employ ion pump to remove the Ca^{2+} (Talarico *et al.*, 2005). The PMCA and the sodium calcium exchanger (NCX) are together the main regulators of intracellular Ca^{2+} concentrations (Carafoli, 1991). Since it transports Ca^{2+} into the extracellular space, the PMCA is also an important regulator of the calcium concentration in the extracellular space (Jensen *et al.*, 2006). The decrease in $[Ca^{2+}]_i$ is due to the activity of Ca^{2+}-clearing mechanisms, such as the plasma membrane Ca^{2+}-ATPase (PMCA) pump, the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump (Carafoli and Brini, 2000) and the plasma membrane Na^+/Ca^{2+} exchanger (NCX) (Blaustein and Lederer, 1999). Ca^{2+} is well recognized as an important regulatory element for many cellular processes; it acts either directly as a 2nd messenger or for the maximum activation of other enzymes in the signal cascade (Putney 1993, Berridge 1997). This approach has already been used to observe $[Ca^{2+}]_i$ in the cells of intact blood vessels isolated in vitro (Kasai *et al.*, 1997; Jaggar *et al.*, 1998; Maruya *et al.* 1999; Ruhelmann *et al.* 2000). However, in these works, only calcium probe (Flura 2/AM) was used. The first goal of the present study was to identify the active ingredients in *V. heterophylla*. Following chemical isolation and identification, some of the major components of *V. heterophylla* turned out to be the triterpenes THO. In this study, we investigated the mechanisms underlying intracellular Ca^{2+} signalling in rat aortic endothelium loaded with the Ca^{2+}-sensitive dye, fura-2/AM.

3 MATERIALS AND METHODS

3.1 Animals: Wistar rats (250 – 350 g) were used for all experiments. Animals were housed under conditions of controlled temperature (20-24°C) and humidity (55% ± 10%). In addition, they had free access to feed (Harlan Teklad, Global diets, Pavia, Italia) and tap water *ad libitum*. The animal handling was under the control of the veterinary surgeon of the University of Pavia. Experimental protocols and

procedures were approved by the institutional Animals Care and Use Committee and the research was approved by the Ethical Committee of the University of Pavia.

3.2 Drug administration: The following drugs were used: Adenosine triphosphate (ATP), 3β-16β,23,29-tetrahydroxyoleane-12-ene (THO), Lanthanum chloride (III) (La^{3+}), Cyclopiazonic acid (CPA), all from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were prepared in distilled water and kept at −4°C. THO was solubilised in distilled water and diluted to the desired concentrations with distilled water just before use. The other compounds were dissolved in distilled water.

3.3. Chemicals Solutions: The Physiological Salt Solution (PSS) had the following composition (in mM) 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4. In Ca^{2+}-free solution, Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Both solutions were titrated to pH 7.4 with NaOH. Fura-2/AM was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). All other chemicals were purchased from Sigma. Medium exchange and administration of agonists or other drugs were performed by removing the bathing medium (2 ml) and adding the desired solution. The medium could be exchanged quickly without producing artefacts in the Fluorescence signal because a small meniscus of liquid remained between the tip of the objective and the facing surface of the cover slip.

3.4 Plant collection and preparation of the extract: The stem bark was collected in February 2010 in the Mokolo (Far North Region) in Cameroon (10° 39.214' N, 14° 24.145' E, 375 m altitude). This region has annual average humidity of 73% and an average temperature of 29° C. This plant was selected on the basis of traditional uses. A sample was identified at the National Herbarium Cameroon (NHC) where a voucher specimen is deposited in number (UICN) EN A1C, B1+2c. The stem bark was cut air-dried and crushed. Two and a half (2.5) kgs of the powder of the plant material was introduced into the methanol, at room temperature. The extraction lasted for 48 hours. After decantation and filtration, the macerate was collected in a volumetric flask. The operation was repeated two times with the solvent. Each extract was concentrated to dryness under reduced pressure using a rotary evaporator (BÜCHI). From this procedure, 175 g of ethyl acetate extract were obtained and preserved at -0.5° C.

3.5 Preparation of fractions and isolation of the molecule: The ^1H NMR spectrum showed a profile that allows detecting the presence of the methyl groups between δ 0.5-2.0 ppm. In addition, we observed a chemical shift at δ 5.25 ppm protons at position 12 and the peak at δ 2.91 ppm proton in position 3, which are characteristic of pentacyclic triterpenes. The ^{13}C NMR spectrum shows signals at δ 121.05 and 145.79 ppm characteristic of vinyl carbons at position 12 and 13 of oleanic triterpenes. The value δ 86.44ppm corresponds to the carbon in position 3 characteristic pentacyclic triterpenes. The complete assignment of the signals was performed by analysis of heteronuclear correlation spectra HSQC and HMBC. Spectrum measurement of heteronuclear correlation HSQC led to the establishment of connections geminal ^1H-^{13}C NMR of the product. It has been shown that connections located at δ 2.91 and 5.25 ppm was attached to CH respectively located at δ 86.44 ppm and 121. The measurement of heteronuclear correlation spectrum observed at long distance showed HMBC correlations between δ 2.91 ppm for proton and carbon at 27.77 (CH_2) 37.67 (CH_2), 64.62 (CH), 23.96 (CH_3) and 25.39 (CH_3) ppm suggesting attachment to the CH_2 groups in C-1 and C-2; to CH in C-5 and CH_3 in C-23 and C-24. The proton chemical shift of the alcohol function is 4.0 ppm in pentacyclic triterpene. This is the case for this compound, in addition, the chemical shift of the carbons in positions 3 and 16 respectively appear δ 76.29 and δ 86.0ppm. This compound is identified for the first time in *Vepris heterophylla* to the best of our knowledge, however, has been isolated and characterized for the first time by Hisashi Kojima and Ogura Hamo in 1989.

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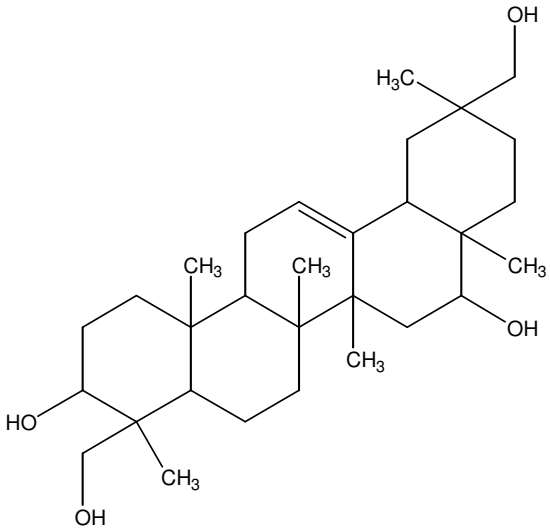

Figure 1: Chemical structure of 3β-16β, 23, 29-tetrahydroxyoleane-12-ene

3.6 Preparation of isolated rat superior aorta rings: Wistar rats were sacrificed by stunning and bleeding. The thoracic and abdominal aorta were dissected out and perfused with Physiological Salt Solution (PSS). The superior aortic artery was removed and cleaned from connective tissue and fat. Rings from rat aorta (0.5 mm) were obtained and placed in Fura-2/AM for 1 hour; the rings were removed and stored in PSS for 30 min, at room temperature (22–24 °C). The preparations were exposed to 3β-16β,23,29-tetrahydroxyoleane-12-ene ((100 μM), Adenosine triphosphate (300 μM) which causes intracellular Ca^{2+} release (Michael *et al.*, 1995), Lanthanum (100 μM) the non-specific calcium channel antagonists, which usually blocks calcium influx and calcium-related metabolic functions such as trans-membrane Ca^{2+} transport within excitable tissues (Fitzpatrick, 1990); Cyclopiazonic acid (10 mM) inhibitor of sarcoplasmic reticulum Ca^{2+}-pump. THO was applied in an extracellular Ca^{2+}-free solution and in an extracellular Ca^{2+}-free solution after depletion of intracellular Ca^{2+}-store by:

1) applying La^{3+} (100 μM) in Ca^{2+}-free solution for 30 minutes, and then wash the ring by extracellular Ca^{2+}-free solution and apply THO in extracellular Ca^{2+}-free solution.

2) applying CPA (10 mM) in Ca^{2+}-free solution for 30 minutes, and then wash the ring by extracellular Ca^{2+}-free solution and apply THO in extracellular Ca^{2+}-free solution,

3) applying ATP 300 μM in Ca^{2+}-free solution for 10 minutes, wash and incubate in Ca^{2+}-free solution for 20 minutes and then wash the ring by extracellular Ca^{2+}-free solution.

Ca^{2+}-free solution can be obtained by both avoiding Ca^{2+} addition and adding EGTA (final concentration 0.5 mM (pH 7.4)

3.7 Intracellular [Ca^{2+}] evaluation: The technique used to evaluate changes in intracellular ($[Ca^{2+}]_i$) in intact endothelium has previously been described (Moccia *et al.*, 2002; Yuly *et al.*, 2010). The aortic ring was opened and loaded with 16 μmol Fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. In situ ECs were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss × 63 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical apertures). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. The exciting filters were mounted on a filter wheel Lambda 10, Sutter Instrument, Novato, Calif., USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot online the fluorescence from 10 to 15 rectangular 'regions of interest' (ROI) enclosing one single cell. $[Ca^{2+}]_i$ was monitored by measuring, for each region of interest, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed 'ratio'). An increase in $[Ca^{2+}]_i$ caused an increase in the ratio. The experiments were performed at room temperature (21–23 °C).

3.8 Data Analysis: Mean values are presented together with standard error of the mean and the whole number of tested cells 'n' or number of experiments. Statistical significance ($p < 0.05$) was evaluated by the Student t test and one-way ANOVA, using Origin Graph, (Microcal Origin 6.0) software version 6.0. Tracings shown in the figures are single cell recording. Emax is the maximal increase in $[Ca^{2+}]_i$ at the highest concentration used. The slope with correlation coefficient was measure by the Fit Linear regression. Values of $P<0.05$ were considered statistically significant.

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4 RESULTS

The superfusion of the strip from rat aorta artery with a Fura-2/AM solution (16 μmol) for 60 min at room temperature, homogeneously increased the fluorescence ratio by 80 ± 19% (n=6) intact endothelium instead 5 ± 22 % without endothelium. The endothelium was impermeable to the dyes we used, so when a strip with intact endothelium was loaded, only the ECs became fluorescent (data not shown). As a control, the baseline levels of $[Ca^{2+}]_i$ under isotonic conditions were also measured (n=4). The measurement began in the presence of 5 mM extracellular Ca^{2+}. At 240 s, the solution was replaced with a Ca^{2+}-free solution (1 mM EGTA), and at 420 s the final washout with solution containing 5 mM extracellular calcium was executed. As expected, the fluorescence ratio F340 nm/F380 nm corresponding to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) decreased after changing to Ca^{2+}-free solution. However, after washout of Ca^{2+}-free solution, there was a sharp increase in $[Ca^{2+}]_i$ over the baseline levels (data not shown). In particular, the F340 nm/F380 nm ratio was significantly increased from 0.198 ± 0.018 (n=4) to 0.352 ± 0.033 at the same time point after washout (n=5). This was then followed by a reduction of the Ca^{2+} levels (0.254 ± 0.021; n=5). This result was reproducible at different cell passages studied, indicating preservation of ECs vitality at least under these conditions. Therefore, there was no damage to the cells by an irreversible increase in $[Ca^{2+}]_i$. The superfusion of the strip from rat aorta ECs with ATP (100 μM) induced an asynchronous increase of calcium in about 70% of the smooth-muscle cells (Figure 2, Table 1).

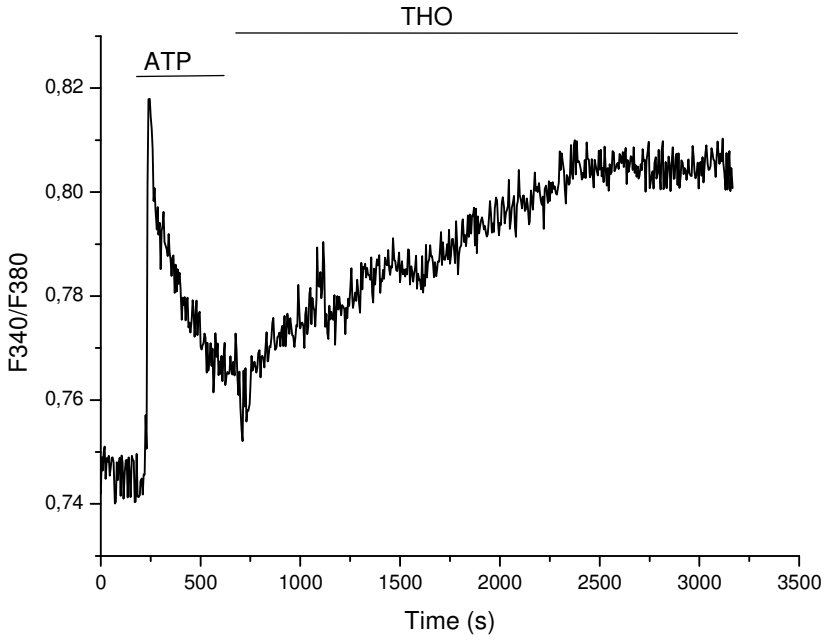

Figure 2: Effect of ATP (300μM) and THO (100μM) on the intracellular Ca^{2+} concentration of aorta ring endothelial cells (single cell tracing)

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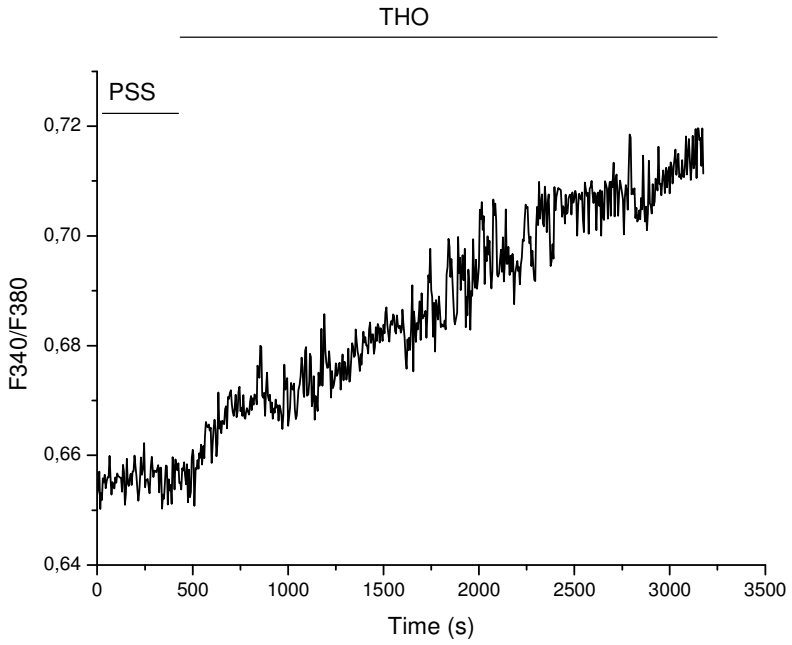

Figure 3: Effect of THO (100μM) on the intracellular Ca^{2+} concentration of aorta ring endothelial cells (single cell tracing)

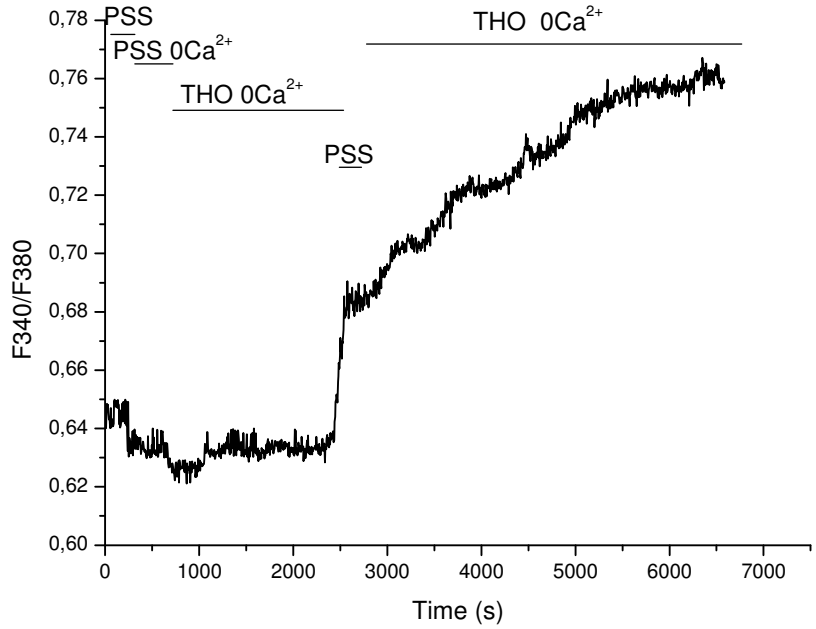

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Figure 4: Effect of THO (100µM) on the intracellular Ca^{2+} concentration of aorting ring endothelial cells in the presence (PSS) and absence of extracellular Ca^{2+} ($0Ca^{2+}$) (single cell tracing)

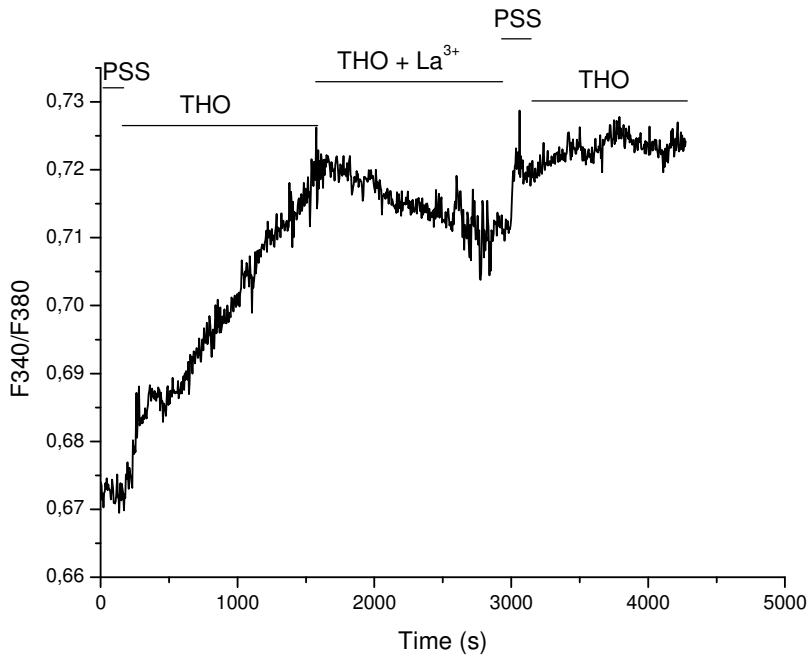

Figure 5: Effect of THO (100µM) on responses to the influx of extracellular Ca^{2+} after (n = 8) pre-treatment of the rings with La^{3+} (100µM).

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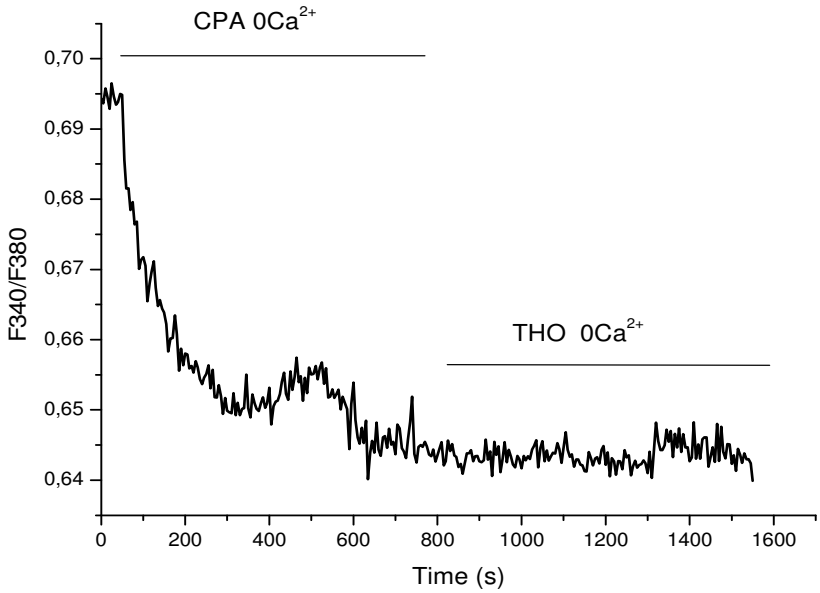

Figure 6: Effect of THO (100μM) on responses to the influx of extracellular Ca^{2+} after (n = 8) pre-treatment of the rings with CPA (10 mM).

In these cells, ATP increased the fluorescence ratio by 17 ± 17% (n=8). As shown in Figure 2, ATP, a receptor agonist, evoked intracellular Ca^{2+} concentration, the decay phase of which was affected by treatment with THO. The amplitude of the Ca^{2+} signal evoked by a high ATP concentration was comparable with the increase in $[Ca^{2+}]_i$ evoked by THO (figure 2), suggesting that THO could indeed evoke a Ca^{2+}-dependent NO synthesis. When applied the strip from rat aorta with a high THO solution (100 μM instead of 50 μM and 25 μM), the fluorescence ratio F340 nm/F380 nm corresponding to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increased from 0.505 ± 0.038 (n=9) to 0.882 ± 0.013, with the Ca^{2+} signal amplitude percentage increased (77.67 %) (Figure 3, Table 1). THO (100 μM) caused a slow, long-lasting increase in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ with a slope = 2.07 and coefficient correlation r = 0.97. In Ca^{2+}-free extracellular solution, the slow increase in $[Ca^{2+}]_i$ was still present, (but with a slope = -1.84, correlation coefficient = -0.24; n = 8, much smaller than control values), suggesting that Ca^{2+} influx is involved (Figure 4, Table 1). When we applied in the incubation medium THO, 20 minutes before the higher magnitude response produced by ATP and 100s before the addition of THO did not provoke any significant modifications of the amplitude effects of THO (data no shown). It is known that at the tested doses of 100 μM for La^{3+}, increase influx of intracellular Ca^{2+} concentration channels can be blocked. In the presence of La^{3+}, the higher slope due to THO was completely abolished as shown in figure 5 and Table 1, suggesting that DMF-induced intracellular influx Ca^{2+} involves the participation of channels calcium. As shown in Figure 5, CPA, a inhibitor of sarcoplasmic reticulum Ca^{2+}-pump, the decay phase was not affected by treatment with THO in Ca^{2+} -free medium, the fluorescence ratio F340 nm/F380 nm corresponding to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increased from 0.6429 ± 0.011 (n=8) to 0.6465 ± 0.013 (Figure 6, Table 1). These results further support the hypothesis that THO-induced intracellular influx Ca^{2+} involves the participation of channels calcium

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Table 1: Emax values for THO in the different experimental intracellular influx of Ca^{2+} conditions

Experimental condition		Emax amplitude	n
	THO (100µM) only	0.85 ± 1.37	9
	ATP (300µM) + THO (100µM)	0.77 ± 1.02	8
CPA (10 mM) + THO (100µM)	THO $0Ca^{2+}$ (100µM)	0.15 ± 0.15	8
THO $0Ca^{2+}$+ THO (100µM)	THO $0Ca^{2+}$ (100µM)	0.19 ± 0.16***	8
	THO (100µM)	0.77 ± 0.25	8
	THO (100µM)	0.65 ± 0.10	8
	THO (100µM) + La^{3+} (100µM)	-0.59 ± 0.11***	8
THO + La^{3+} (100µM)	THO (100µM)	0.21 ± 0.13	8

n, number of Cells. Values are mean ± S.E.M., *** P<0.001 vs. endothelium cells incubated in PSS in the same experiment condition or PSS $0Ca^{2+}$

5 DISCUSSION

The ECs loaded with Fura-2/AM were unambiguously recognizable by their shape. The observation of an increase in $[Ca^{2+}]_i$ in the ECs upon incubation of the aorta ring in high ATP (300 µM) suggests that calcium ions diffused from the smooth-muscle cells to the endothelial cells' cytoplasm. Although some endothelial cells have alpha-2 adrenoceptors, an alpha-1 agonist does not stimulate an endothelium-dependent response in rat aorta artery (Angus and Cocks, 1986). The observed $[Ca^{2+}]_i$ increase in endothelial cells suggests, then again, that calcium ions diffused from the smooth muscle to the ECs cytoplasm. However, in the case of an alpha-1 adrenoceptor stimulation of the smooth-muscle cells, in addition to calcium, other water-soluble small molecules, such as the intracellular second messenger IP_3, can diffuse through the gap junctions and secondarily cause an increase in calcium (Sandow and Hill, 2000; Carter *et al*., 1996). Calcium or IP_3 did not diffuse from the smooth-muscle cells to all the ECs. Hence, only a few clusters of ECs within the observed field are influenced by the calcium increase in the smooth-muscle cells. Upon THO stimulation, an increase in $[Ca^{2+}]_i$ was observed in plus 70% of the endothelial cells. Therefore, the indirect influence of THO on only a few clusters of ECs could be related to this fact. An alternative explanation for this is that, although the ECs are well homo-cellularly coupled together, only some ECs are heterocellularly coupled to the smooth-muscles (Bény, 1999). THO caused an increase in $[Ca^{2+}]_i$ in the ECs. This could activate the NO synthase and the production of endothelium-derived hyperpolarizing factor (EDHF) (Chen and Suzuki 1990, Förstermann *et al.* 1991). It is well known that activation of constitutive endothelial NOS may be Ca^{2+} dependent. THO caused a slow, long lasting increase in the $[Ca^{2+}]_i$ of aortic ECs. Such a slow $[Ca^{2+}]_i$ increase was very limited in Ca^{2+}-free extracellular medium. The slope in Ca^{2+}-free extracellular medium was negative of the slope in normal solution. This molecule isolated in the bark of trunk of *Vepris heterophylla* (Rutaceae), which the effects on the increase of intracellular calcium are prove could justify an empirical use by traditional healers in the treatment of arterial hypertension as a medicinal plant. It is supposed that the endothelium-dependent vasodilatation is caused by a decrease of $[Ca^{2+}]_i$ in the smooth-muscle cells in response to the relaxing factors released by the ECs (Somlyo and Somlyo, 1994). In this way, when calcium increases in the ECs, it simultaneously decreases in the coupled smooth-muscle cells. The observation that the decrease of $[Ca^{2+}]_i$ is weak in the smooth-muscle cells is consistent with the small decrease in $[Ca^{2+}]_i$ caused by a bradykinin induced relaxation observed in the porcine coronary artery (Hirano and Kanaide, 1993). This points out that a calcium decrease in the smooth-muscle cells is probably not sufficient to explain the effect of endothelium-dependent relaxing factors on these cells (Hirano and Kanaide, 1993). This result suggests THO causes a slow influx of extracellular Ca^{2+}. Release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanisms. Ca^{2+} is known as an important regulatory element for many cellular processes; it acts either directly as a 2nd messenger or for the maximum activation of other enzymes in the signal cascade (Putney, 1993, Berridge, 1997). THO (10^{-4} M), administrated in Ca^{2+} free medium did not increase intracellular influx Ca^{2+}. What suggest that THO would act on the calcium

channels by stimulating their openings, thus allowing a massive entry of Ca^{2+} into the cell? The lack of Ca^{2+} entry was verified by the failure of ATP to produce increase amplitude in the aortic rings in the absence of extracellular Ca^{2+} (data not shown). As shown on Figure 3, in Ca^{2+}-free extracellular solution, the slow increase in $[Ca^{2+}]_i$ was still present. To verify if the slow increase in $[Ca^{2+}]_i$ did not involves the sarcoplasmic reticulum Ca^{2+}-pump, the preparations were pre-treated with Cyclopiazonic acid (10 mM) inhibitor of sarcoplasmic reticulum Ca^{2+}-pump as shown in figure 5. This observation might explain our result that, in the presence of sarcoplasmic reticulum Ca^{2+}-pump inhibition, THO did not induced increased intracellular Ca^{2+} concentration, The lack of effect of SERCA pump inhibition on THO-induced Ca^{2+} might be explained shown that THO did not used the intracellular Ca^{2+} in this case less Ca^{2+} has to be extruded out of the cell. To determine if THO-induced intracellular influx Ca^{2+} involves the participation of channels calcium, the preparations were pre-treated with La^{3+} the non-specific calcium channel antagonists, which usually blocks calcium influx and calcium-related metabolic functions such as trans-membrane Ca^{2+} transport within excitable tissues (Fitzpatrick, 1990), inhibited higher amplitude due to THO-induced Ca^{2+} release as illustrated in Figure 4. Negative effects of lanthanum on cells are attributed to its blockage of Ca^{2+}-channel on the cell membrane, and on this basis, a higher concentration of lanthanum (100 μM) has been extensively used to inhibit Ca^{2+} influx to investigate various Ca^{2+}-dependent cellular processes in rat aorta (Jan *et al.*, 1998; Lewis *et al.*, 1998; Geitmann and Cresti, 1998; Friedmann *et al.*, 1998).

6 CONCLUSION

In conclusion, the present studies identified THO as some of the active ingredient in *V. heterophylla* and verified its intracellular Ca^{2+} concentration effect. THO caused a slow, long lasting increase in the $[Ca^{2+}]_i$ of aortic ECs. THO-induced intracellular influx Ca^{2+} involves the participation of channels calcium. Such a slow $[Ca^{2+}]_i$ increase was very limited in Ca^{2+}-free extracellular medium. Mechanistic studies suggested vasorelaxation via increase intracellular Ca^{2+} concentration being the likely underlying mechanism. Vascular endothelium and related vasorelaxation mediators play small roles. These findings may be helpful in the establishment of THO as a potential antihypertensive agent, elucidation of its pharmacological actions and its further development as a therapeutic agent.

7 ACKNOWLEDGEMENTS

The first author received a research fellowship from CICOPS, Università degli studi di Pavia, Italy.

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