Effects of Total Flavonoids of *Hippophae Rhamnoides* L. on Intracellular Free Calcium in Cultured Vascular Smooth Muscle Cells of Spontaneously Hypertensive Rats and Wistar-Kyoto Rats*

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ABSTRACT **Objective:** To explore the effects of total flavonoids of *Hippophae rhamnoides* L. (TFH), quercetin (Que) and isorhamnetin (Isor) on the intracellular free calcium ($[Ca^{2+}]_i$) in vascular smooth muscle cells (VSMC) of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). **Methods:** Fluo 3-acetoxymethylester(Fluo-3/AM) was used to observe the effects of TFH (100mg/L) and its essential monomers, namely Que (10^{-4}mol/L) and Isor (10^{-4}mol/L) on changes of $[Ca^{2+}]_i$ in cultured SHR and WKY VSMC (abbr. to Ca-SHR & Ca-WKY) following exposure to high K^+, norepinephrine (NE) and angiotensin Ⅱ (Ang Ⅱ), and to compare with the effects of verapamil (Ver). **Results:** (1) TFH, Que and Isor had inhibitory effects on resting Ca-SHR ($P<0.05$), but had no significant effects on Ca-WKY ($P>0.05$). (2) High K^+ could increase Ca-SHR more significantly than Ca-WKY ($P<0.05$); TFH, Que and Isor could inhibit the elevation of $[Ca^{2+}]_i$ induced by high K^+-depolarization, with the effects similar to that of Ver, and the effect on Ca-SHR was more significant than that on Ca-WKY ($P<0.05$). (3) NE and Ang Ⅱ could increase Ca-SHR more significantly than Ca-WKY ($P<0.05$), TFH, Que and Isor had remarkably inhibitory effect on the elevation of Ca-SHR and Ca-WKY induced by NE or Ang Ⅱ. (4) In the absence of extracellular Ca^{2+}, TFH, Que and Isor also had certain inhibitory effect on Ca-SHR and Ca-WKY induced by NE, and the effect on the former was more significant than that on the latter($P<0.05$). **Conclusion:** TFH, Que and Isor might decrease the levels of $[Ca^{2+}]_i$ in VSMCs by blocking both voltage-dependent calcium channels (VDC) and receptor-operated calcium channels (ROC) in physiological or pathological state, which may be one of the important mechanisms of their hypotensive and protective effects on target organs in patients with hypertension.

KEY WORDS total flavonoids of *Hippophae rhamnoides* L., spontaneously hypertensive rats, Wistar-Kyoto rats, vascular smooth muscle cells, intracellular free calcium

Essential hypertension (EH) has become one of the main diseases that endanger human health in modern society. It has been a key project for scholars at home and abroad to find out how to effectively prevent and cure hypertension and lesions in its target organs.

Total flavonoids of *Hippophae rhamnoides* L. (TFH), a natural flavonoids, is extracted from the fruit of wild plant *Hippophae rhamnoides* L., the great mass of which is quercetin (Que) and isorhamnetin (Isor). Research has found that TFH has dilatation of vascular and hypotensive effect, and meanwhile could protect and reverse the lesions of target organs induced by EH, but the mechanism is not yet clear now[1].

Since the effect of TFH on intracellular free calcium ($[Ca^{2+}]_i$) in vascular smooth muscle cells (VSMC) of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) in either pathological or physiological condition and on calcium channels has not been reported so far, in order to explore the mechanisms of the protection on target organs and hypotensive action of TFH, the authors observed, by using Fluo 3-acetoxymethylester (Fluo-3/AM), the effects of TFH and its monomers, Que and Isor, on changes of $[Ca^{2+}]_i$ in cultured VSMCs of SHR and WKY (abbr. to Ca-SHR & Ca-WKY) after exposing the cells to high K^+, norepinephrine (NE) and angiotensin Ⅱ

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(Ang Ⅱ), and compared the effects with that of verapamil (Ver).

METHODS

Drugs and Reagents

THF and Isor were gained from West China Medical University (purity > 98%); Que, Ver, NE, N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (HEPES), Fluo-3/AM, angiotensin Ⅱ (Ang Ⅱ), ethylene glocol-bis-(2-aminoethylether)-N, N, N'N'-tetraacetic acid (EGTA), Eagle's minimum essential medium (MEM) culture media were purchased from Sigma Co.; and A23187 from CNI Co., USA. Other reagents were all analytically pure, purchased from the home market.

Cultivation of VSMCs

Male SHR and WKY, aged 25 weeks, provided by Shanghai Institute of Hypertension, were housed in cages under controlled temperature and humidity and given free access to standard forage and water. The investigation was performed according to the European Community Guidelines for animal ethical care and use of experimental animals. Their systolic blood pressure, measured by the standard tail-cuff method before experiment, conformed to the required values for animal models at this age (213±6 mmHg, n=12).

Cell isolation and culture were conducted referring to literature[2]. Primary VSMC obtained from the thoracic aorta of adult SHR and WKY were cultured by tissue transplant method. Briefly, the thoracic aorta was longitudinally opened, and after the endothelium was removed, the median membrane was cut into small pieces 1 mm × 1 mm in size. Then, the tissue pieces were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and antibiotic-antimycotic agents. Fusiform or long fusiform cells growing along the wall of incubator could be seen after it was cultured for about one week. When VSMCs emerged all over the bottom, they were digested by 0.25% trypsin for about 1 min. After the primary culture, the culture of passage cells would be carried out when cell culture became confluent in 5 to 6 days, manifesting typical cusp and vallecula. The medium was changed initially after 24 hrs and then every 3 to 4 days. The cells of the 3rd to 8th generation were used in the study.

Grouping and Managing of VSMCs Samples

The samples were divided into four groups: the blank group (resting group, 16 samples), the agitated group, the tested group and the control group. All the cell samples in the tested group, which were divided into 3 subgroups, 6 in each, were incubated firstly with the testing drugs, namely TFH (100 mg/L), Que (10^{-4} mol/L) and Isor (10^{-4} mol/L), respectively for 6 min. Then the agitating agents (high K^+, NE and Ang Ⅱ) were added separately to the cell samples (two for each agent). For samples in the agitated group, which was divided into 3 subgroups managed by the 3 agitating agents respectively with no use of testing drug, while the samples in the blank group were treated merely with testing drugs respectively but with no agitating agent added into it. As for those in the control group, they were treated with the same scheme as those used for the tested group except that Ver (10^{-4} mol/L) was used to substitute the testing drug.

Measurement of $[Ca^{2+}]_i$ in VSMCs

Referring to literatures[3,4], VSMCs (2.5×10^4 cells/ml) grown and confluent on 13 mm × 25 mm cover-glass were load-managed with Fluo-3-AM. In the presence of extracellular Ca^{2+}, the balanced salt solution containing Ca^{2+} (BBS, mmol/L: NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.25, glucose 10, HEPES 20, and EGTA 1, pH 7.4) was used, while in the absence of extracellular Ca^{2+}, the balanced salt solution without Ca^{2+} was used instead (mmol/L: NaCl 135, KCl 5, $MgCl_2$ 1, glucose 10, and HEPES 20, pH 7.4). Intensity of fluorescence was detected by RF-5000 pc type fluoremeter (Japan) at 37℃, with the parameters set on EX Bandbass 5 nm, EM Bandbass 10 nm; λex 505 nm, λem 526nm, response 2 s.

Fluorescence (F) in resting state of samples was measured at first, and the value was taken as the baseline. Then, A23187 (10^{-5} mol/L) was added to read the maximum fluorescence (Fmax). Finally, fluorescence minimum (Fmin) was measured after adding EGTA (5mmol/L). The concentration of $[Ca^{2+}]_i$ can be calculated according to the following formula: Concentration of $[Ca^{2+}]_i = Kd(F-Fmin)/(Fmax-F)$. In the formula Kd = 450 nmol/L, which was the disintegration constant between Ca^{2+} and Fluo-3/AM.

Statistical Analysis

The results were expressed as $\bar{x} \pm s$, t-test was used in analyzing the comparison between the two groups, and data among groups were compared with analysis of variance between groups (ANOVA).

RESULTS

Effects on Ca-SHR and Ca-WKY in the Blank Group

There was no significant difference between the levels of Ca-SHR and Ca-WKY in single layer cultures of the blank group either in condition of presence or absence of extracellular Ca^{2+} ($P>0.05$). However, either Ca-SHR or Ca-WKY with extracellular Ca^{2+} condition absent was lower than those in the presence of extracellular Ca^{2+} condition ($P<0.05$).

The level of Ca-SHR was decreased by 10.2±2.1%, 7.0±1.3 %, 8.3±2.0%, and 10.8±1.7% after it was treated by Que, Isor, TFH and Ver respectively in the presence of extracellular Ca^{2+}, and turned into 11.0±1.9%, 6.6±1.6%, 8.6±1.8%, and 10.4±2.1% respectively in the absence of extracellular Ca^{2+}, suggesting that TFH, Que, Isor and Ver had certain inhibitory effect on resting Ca-SHR ($P<0.05$) either when extracellular Ca^{2+} was present or absent, and showed no significant difference between the effects under the two conditions ($P>0.05$). See Figure 1.

Que, Isor, TFH and Ver showed no inhibitory effect on single layer cultured Ca-WKY either in condition of presence or absence of extracellular Ca^{2+} ($P>0.05$).

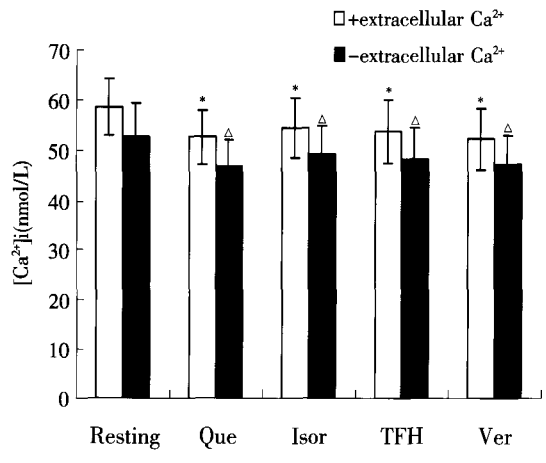

Figure 1. Effects of Que, Isor, TFH and Ver on Resting Ca-SHR in the Presence and Absence of Extracellular Ca^{2+} ($\bar{x}\pm s$, $n=6$)

Notes: * $P<0.05$, compared with the resting $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}; △ $P<0.05$, compared with the resting $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}

Effects on the Elevation of VSMC $[Ca^{2+}]_i$ Induced by High K^+-Depolarization

See Table 1. The results indicated that the voltage-dependent calcium channels were opened in depolarization induced by high K^+ (KCl 80 mmol/L), and levels of Ca-SHR and Ca-WKY were both increased, the increment was 481.9±29.6% and 352.6±33.7% respectively, the effect on the former were more significant than that on the latter ($P<0.05$).

Table 1. Effects of High K^+ on Ca-SHR and Ca-WKY (nmol/L, $\bar{x}\pm s$, $n=6$)

Group	$[Ca^{2+}]_i$	
	Resting	KCl
Ca-SHR	57.4±5.2	335.4±9.7*
Ca-WKY	58.4±5.6	259.5±10.8

Note: * $P<0.05$, compared with the level of Ca-WKY

When VSMC was pretreated with Que, Isor, TFH and Ver respectively before adding KCl, the elevated levels of Ca-SHR and Ca-WKY were decreased by 87.2±8.2%, 37.6±6.3%, 80.1±8.8%, 84.6±7.9% and 63.4±7.2%, 18.6±4.1%, 57.8±5.2%, 64.9±6.2% respectively, showing significant inhibitory effects by these testing drugs ($P<0.01$), and their effect on Ca-SHR was more significant than that on Ca-WKY ($P<0.05$) respectively. Comparison of the effects between the testing drugs at the same dose in the same condition showed that significant difference presented between Que and Isor or TFH ($P<0.05$) but not between Que and Ver. See Figure 2.

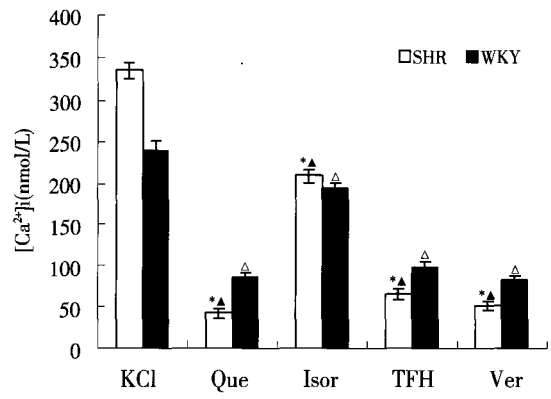

Figure 2. Inhibitory Effects of Que, Isor, TFH and Ver on High K^+ Induced $[Ca^{2+}]_i$ Elevation ($\bar{x}\pm s$, $n=6$)

Notes: * $P<0.01$, compared with Ca-SHR treated by KCl alone; △ $P<0.01$, compared with Ca-WKY treated by KCl alone; ▲ $P<0.05$, compared with Ca-WKY treated by corresponding agent

Effects on the Elevation of VSMC $[Ca^{2+}]_i$ Induced by NE

NE (10^{-6} mol/L) showed a significant elevating effect on Ca-SHR and Ca-WKY in the presence of extracellular Ca^{2+} ($P<0.01$), the increment was 378.8±27.8% and 221.7±31.2% respectively, with the former higher than the latter ($P<0.05$). See Table 2.

Table 2. Effects of NE on VSMC $[Ca^{2+}]_i$ in the Presence of Extracellular Ca^{2+} (nmol/L, $\bar{x}\pm s$, $n=6$)

Group	$[Ca^{2+}]_i$	
	Resting	NE
Ca-SHR	57.7±5.7	276.9±10.7*△
Ca-WKY	58.1±5.9	185.9±11.4*

Notes: * $P<0.01$, compared with the same group in the resting condition; △ $P<0.05$, compared with WKY level

In the absence of extracellular Ca^{2+}, NE also showed an obvious elevating effect on $[Ca^{2+}]_i$ ($P<0.01$). The increment of Ca-SHR and Ca-WKY was 249.1±36.2% and 112.4±32.6% respectively, with the effect on the former more obvious than that on the latter($P<0.05$). See Table 3.

Table 3. Effects of NE on VSMC $[Ca^{2+}]_i$ in the Absence of Extracellular Ca^{2+} (nmol/L, $\bar{x}\pm s$, $n=6$)

Group	$[Ca^{2+}]_i$	
	Resting	NE
Ca-SHR	51.2±5.6	179.2±11.4*△
Ca-WKY	50.1±5.9	105.2±10.7*

Notes: The same as Table 2

Que, Isor and TFH had a significant inhibitory effect on the level of Ca-SHR and Ca-WKY elevated by NE in the presence or absence of extracellular Ca^{2+} condition ($P<0.01$ or $P<0.05$), the effect on Ca-SHR was much more obvious than that on Ca-WKY ($P<0.05$ or $P<0.01$). See Figure 3, 4.

Effects on the Elevation of VSMC $[Ca^{2+}]_i$ Induced by Ang Ⅱ

See Table 4. The results indicated that Ang Ⅱ (10^{-6} mol/L) had a significant elevating effect on Ca-SHR and Ca-WKY($P<0.01$) with the increment of 493.9±33.2% and 348.7±36.2% respectively. The effect on Ca-SHR was significantly higher than that on Ca-WKY ($P<0.05$).

Que, Isor and TFH could decrease Ang Ⅱ induced elevation of Ca-SHR by 62.0±4.6%, 48.0±4.8% and 51.6±5.0% ($P<0.01$)

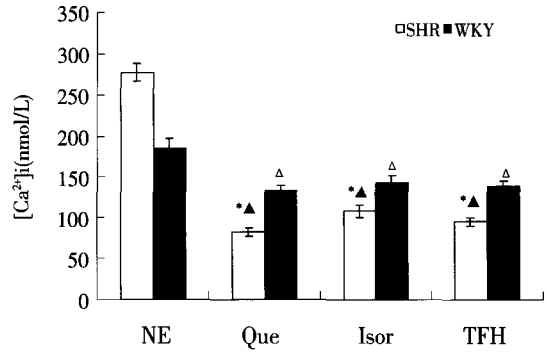

Figure 3. Inhibitory Effects of Que, Isor and TFH on NE (10^{-6} mol/L) Induced Elevation of Ca-SHR and Ca-WKY in the Presence of Extracellular Ca^{2+} ($\bar{x}\pm s$, $n=6$)

Notes: * $P<0.01$, compared with Ca-SHR treated by NE alone; △ $P<0.05$, compared with Ca-WKY treated by NE alone; ▲ $P<0.05$, compared with Ca-WKY treated by corresponding agent

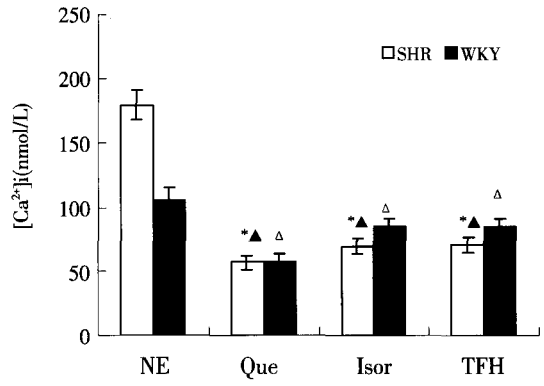

Figure 4. Inhibitory Effects of Que, Isor and TFH on NE (10^{-6} mol/L) Induced Elevation of Ca-SHR and Ca-WKY in the Absence of Extracellular Ca^{2+} ($\bar{x}\pm s$, $n=6$)

Notes: The same as Figure 3

Table 4. Effects of Ang Ⅱ on SHR or WKY VSMC $[Ca^{2+}]_i$(nmol/L, $\bar{x}\pm s$, $n=6$)

Group	$[Ca^{2+}]_i$	
	Resting	Ang Ⅱ
Ca-SHR	58.6±6.9	345.7±11.2*△
Ca-WKY	57.2±6.2	257.4±12.3*

Notes: The same as Table 2

and that of Ca-WKY by 46.0±5.1%, 22.3±4.2% and 25.2±4.0% ($P<0.05$) respectively, with the effect on the Ca-SHR more significant than that on the Ca-WKY ($P<0.01$). See Figure 5.

DISCUSSION

At present, TFH is widely used in clinical practice in treating ischemic heart disease and es sential hypertension[5] . Recent researches have

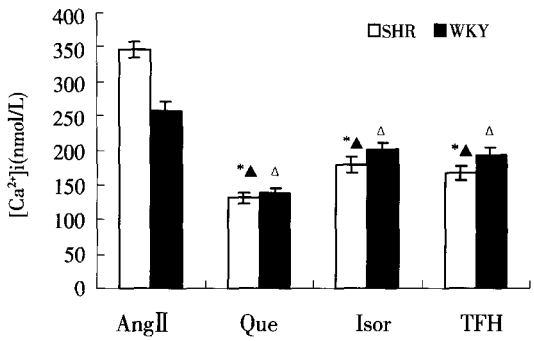

Figure 5. Effects of Que, Isor and TFH on Ang Ⅱ Induced Elevation of Ca-SHR and Ca-WKY ($\bar{x}\pm s$, $n=6$)

Notes: $^{*}P<0.01$, compared with Ca-SHR treated by Ang Ⅱ alone; $^{\triangle}P<0.05$, compared with Ca-WKY treated by Ang Ⅱ alone; $^{\blacktriangle}P<0.01$, compared with Ca-WKY treated by corresponding agent

found that[1] TFH has a protective effect on target organs in hypertension and could reverse the hypertensive injury in them, but the mechanisms of the effects on molecular cytological level is still not clear[6]. It has been found in previous studies[5,6] by the authors and other scholars that TFH has an inhibitory effect on angiotensin-converting enzyme, which could decrease the level of $[Ca^{2+}]_i$ in erythrocyte[5,6]. But so far no report about its effects on $[Ca^{2+}]_i$ of VSMC under pathologic and physiologic conditions has yet been published.

The patients with EH failed to control Ca^{2+} in cells. SHR is the most typical experimental animal model with genetic hypertension, while WKY is the most commonly used experimental model rat. The cultured VSMC is not only a relatively stable and uniform population but also could be directly used in intervention research, the result obtained from which could basically reflect the true state of the patients.

In this study the effects of TFH and its essential monomers on Ca-SHR and Ca-WKY under various conditions were investigated and compared, using the calcium fluorescent probe fluo-3/AM. The results showed that Que, Isor and TFH have no remarkable effect like that of Ver on resting Ca-WKY in conditions either with extracellular Ca^{2+} present or absent, which was in accord with that reported in literature[7] and also in accord with the result reported by Durate J, et al[8]. In his report, Durate J indicated that Que has no significant relaxing effect on resting vascular tension of aortic vascular strip. The results suggested that TFH and its essential monomers have no significant influence on the dynamic regulation on the balance of $[Ca^{2+}]_i$ or on its passive diffusion and active transportation in VSMC of WKY (the experimental animal in physiological manner) in the resting state with normal ranged extracellular Ca^{2+}. But it has been found that TFH and its essential monomers, similar to Ver, did show a mild inhibitory effect on those in VSMC of SHR (the experimental animal in spontaneous pathological manner) either with extracellular Ca^{2+} present or not. These facts suggest that under pathological condition, even in resting state, there are regulative disorder and imbalance of cellular Ca^{2+} homeostasis in SHR, and the results are the same as those reported by others scholars[9].

Our previous study proved that the existing voltage-dependent calcium channel (VDC) and receptor-operated calcium channel (ROC) in VSMC are the main pathways for the influx of Ca^{2+}[10]. The increase of $[Ca^{2+}]_i$ may be induced by high extracellular K^+, NE or Ang Ⅱ due to the high extracellular K^+ manner which could, by way of depolarizing cell membrane and activating VDC, cause a large amount of extracellular Ca^{2+} to enter into cells[11]; NE could promote the release of intracellular Ca^{2+} storage mediated by α_1 receptor, and in case of presence of extracellular Ca^{2+}, could increase $[Ca^{2+}]_i$. Moreover, by way of inducing the influx of Ca^{2+}[12]. Ang Ⅱ could promote both the influx of extracellular Ca^{2+} and the release of intracellular Ca^{2+} storage through binding itself with its receptor[13].

In this study, it was shown that Que, Isor and TFH could significantly inhibit Ca-SHR and Ca-WKY induced by high K^+ depolarization, and the effect on the former is much stronger and hereby we reached the same conclusion as has been reported in literature[14].

The effect of TFH and its essential monomers in antagonizing the vasoconstriction effect induced by KCl were similar, they could definitely inhibit the VDC in VSMC of SHR and WKY, so as to decrease $[Ca^{2+}]_i$ in VSMC, and the inhibition is more apparent on SHR when the rat is in the pathological state.

NE and Ang Ⅱ are very strong vasoconstrictors, which play a very important role in the pathogenesis of EH by activating ROC of cell membrane to cause influx of extracellular Ca^{2+} and release of intracellular Ca^{2+} storage. It has been reported that TFH and Que could significantly antagonize the contractive response of aortic strips in rabbits and rats induced by NE, and similar anti-

contraction effect has also been found in the condition when extracellular Ca^{2+} is absent, suggesting that TFH may have certain inhibitory effect on ROC.

Our study has found that TFH and its essential monomers could inhibit the increase of Ca-SHR and Ca-WKY induced by NE and Ang Ⅱ to a certain extent, and the effect on the former is stronger than that on the latter. Meanwhile, they can inhibit the release of intracellualar Ca^{2+} storage induced by NE when extracellular Ca^{2+} is absent, illustrating from the cytological level that TFH could decrease through ROC free intracellular Ca^{2+} in physiological or pathological state, especially in the pathological state. This effect may be related to the mechanism of its hypotensive action.

It has been found that TFH and its essential monomers could decrease the $[Ca^{2+}]_i$ of VSMCs by double-blocking both VDC and ROC in pathological and physiological state, especially in the pathological state, which is possibly one of the mechanisms of its hypotensive effect. The elevation of $[Ca^{2+}]_i$ induced by vascular regulators like NE, Ang Ⅱ etc., through the mediation by corresponding receptors, could not only increase vascular reactivity in EH patients but also trigger off vascular smooth muscles and proto-oncogene's expression in myocardial cells, playing a vital role in causing atherosclerosis and some pathological proliferative and hypertrophic changes of myocardial cells[15].

At present the effect is not satisfactory when calcium antagonists alone are used to reverse EH caused target organ damages, which is possibly due to the fact that conventionally used calcium antagonists could block VDC but not ROC. Therefore, to doubly block both VDC and ROC and to maintain the $[Ca^{2+}]_i$ steady state might be one of the important mechanisms of TFH and its essential monomers in protecting the target organs in EH patients.

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