Madagascine activates AMPK to induce vasodilatation

(Madagascine は AMPK を活性化して血管拡張を誘発する)

氏名　　呂博超

所属　山口大学大学院医学系研究科

応用医工学系専攻　　分子細胞生理学講座

令和　2 年　11　月

Contents

ABSTRACT

BACKGROUND AND PURPOSE

Madagascine (3-isopentenyloxyemodin), is found to have more potent biological activities than the parent compound emodin. Madagascine can be chemically synthesized or purified from several *Rhamnus* species. This study was designed to characterize the effects of madagascine on activation of AMP-activated protein kinase (AMPK) induced vasodilatation.

EXPERIMENTAL APPROACH

The effects of madagascine on vasoconstriction induced by K^+ and on Ca^{2+}-independent vasoconstriction induced by sphingosylphosphorylcholine (SPC) which has a pivotal role in vasospasm were studied. The contractile force and intracellular Ca^{2+} were respectively studied using a fura-2 fluorometer. The potential mechanism was studied using endothelial cells and vascular smooth muscle (VSM) cells respectively.

KEY RESULTS

The constriction of isolated rat mesenteric resistance arteries with intact endothelium induced by 40 mM K^+ was significantly inhibited by madagascine (0.3-100μM) and the inhibition was abolished by epithelial nitric oxide synthase (eNOS) inhibitor L-NAME and AMPK inhibitor compound C. Madagascine also significantly inhibited the constriction of porcine VSM induced by SPC and the inhibition was also abolished by compound C. Madagascine significantly increased the phosphorylation of eNOS in endothelial cells while decreasing the phosphorylation of myosin phosphatase target subunit 1 (MYPT1) in HCASMCs. These madagascine-induced regulatory effects were abrogated using small interfering RNA knockdown of AMPK.

CONCLUSIONS AND IMPLICATIONS

Madagascine exerted vasodilatation through activating AMPK, leading to the activation of eNOS in epithelium and inhibition of ROCK/MYPT1 in VSM. This study suggests the potential value of madagascine in amelioration of hypertension and vasospasm.

Keywords: Madagascine/ AMPK/ eNOS/ Hypertension / Vasospasm

Abbreviations

HUVECs, Human umbilical vein endothelial cells; SPC, sphingosylphosphorylcholine; VSM, vascular smooth muscle; MRAs, mesenteric resistance arteries.

INTRODUCTION

Vascular vasodilatation has beneficial effect on improvement of cardiovascular diseases such as essential- and renal-parenchymal-disease-related hypertension, cardiac infarction, vascular remodeling and congestive heart failure (Hisham *et al*., 2012; Kumar *et al*., 2012; Machino *et al*., 2014). Vascular tone is determined by the vasoconstriction which is related to a complex interplay of vasodilator and vasoconstrictor substances (Wang et al., 2011). The drugs have directly or indirectly vasodilatory effect including inhibitors of renin-angiotensin system, diuretics, nitrates, antagonists of adrenergic receptors, calcium channel blockers. (Cox *et al.*, 2002; Ji, 2013; Kondo *et al.*, 1979; Salihi, 2013) which possess the potential in improvement of cardiovascular diseases.

According to recent research we find that AMP-activated protein kinase (AMPK) is a new therapeutic target for vasodilatation. AMPK is activated by the phosphorylation of LKB1 and CAMKK in endothelial cells (Stahmann *et al.*, 2006). The activation of AMPK leads to vasodilatation which is regulated by the phosphorylation of epithelial nitric oxide synthase (eNOS) at site Ser1177 in epithelium and the direct inhibitory effect on vascular smooth muscle (VSM) constriction (Bradley et al., 2010; Reihill et al., 2007; Shuangxi et al., 2011). Phosphorylation of 20-kDa myosin light chain (MLC) leads to smooth muscle constriction, which is regulated by both Ca^{2+} dependent and Ca^{2+} independent mechanisms (Somlyo *et al.*, 1994). The activation of AMPK induce the inhibitory effect on Rho-associated protein kinase (ROCK) which mediates Ca^{2+} independent VSM constriction through inhibiting myosin phosphatase via phosphorylation of myosin phosphatase target subunit 1 (MYPT1)(Shuangxi *et al.*, 2011; Somlyo, 2002). SPC is a phospholipid mediator in blood plasma and it exert multifunctional role in cell physiological regulation (Satoshi et al., 2002). Sphingosylphosphorylcholine (SPC) generated by N-deacylation of sphingomyelin which is one of the most abundant lipids in cell membrane (Yue et al., 2015). SPC-mediated activation of ROCK has been proved to be involved in pathogenesis of vasospasm (Fumiaki *et al.*, 2002; Somlyo, 2002). However, the relationship between AMPK activation and vasospasm induced by SPC remains unclear.

Madagascine (3-isopentenyloxyemodin) is a natural compound containing an anthraquinone core linked to a 3, 3-dimethylallyoxy chain (Figure 1). The trivial name of madagascine derives

from the natural source, *Harungana madascariensis* Poir, from which it was isolated and structurally characterized for the first time (Epifano et al., 2013; Ritchie et al., 1964). Subsequently madagascine has also been obtained from several other natural sources including medicinal plants belonging to *Rhamnus spp* (Delle Monache et al., 1987; Iinuma et al., 1995; Mbaveng et al., 2008). Natural compounds containing an anthracene are widely distributed in the plant kingdom and madagascine emerged as one of the most promising compound from a pharmacological point of view (Epifano *et al.*, 2013). Madagascine is found to have diverse biological effects, including anticancer effects, antioxidant and antimicrobial (Ee et al., 2011; Epifano et al., 2013; Mbaveng et al., 2008).

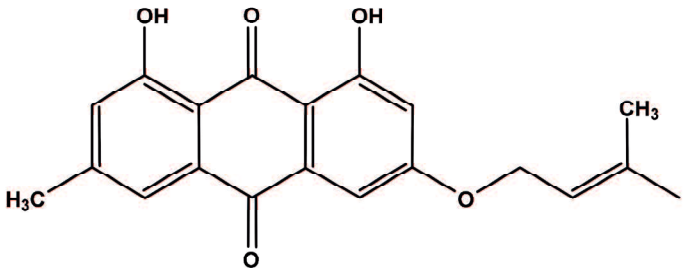

Figure 1. Chemical structure of madagascine.

Emodin has been reported to induce the activation of AMPK in skeletal muscle and liver cells (Song *et al.*, 2013; Subramaniam *et al.*, 2013). Under the same experimental conditions, the biological activity of madagascine was more potent and safe than the activity of emodin (Ee *et al.*, 2011; Mbaveng *et al.*, 2008). According to our pre-experiments, the present study was designed to characterize the vasodilatory effect of madagascine on both normal constriction which is regulated by Ca^{2+} dependent mechanisms and sphingosylphosphorylcholine induced vasospasm in *ex vivo* and *in vitro*. The isolated rat mesenteric resistance arteries (MRAs) were used to investigate the effects of madagascine-mediated activation of AMPK on vasoconstriction. The porcine coronary arteries were used to investigate the effects of madagascine-mediated activation of AMPK on abnormal constriction induced by SPC because porcine coronary arteries are cholesterol-enriched and cholesterol promotes SPC-induced the Ca^{2+} independent of VSM constriction (Noriyasu *et al.*, 2006). Human umbilical vein endothelial cells (HUVECs) and human coronary artery smooth muscle cells (HCASMCs) were used to investigate the vasodilator properties of madagascine respectively.

MATERIALS AND METHODS

MATERIALS

Animals

The experimental protocol was approved by Dalian Medical University Animal Care and Ethics Committee at June 8th, 2012, and all animals used were maintained in accordance with National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85-23, revised 1985). Fifty Male Wistar rats (weighing 200-300 g) were obtained from Experimental Animal Center, Dalian Medical University. (Certificate of Conformity: No. SCXK (Liao) 2008-0002). The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23°C, 12h/12h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks before experimentation. All rats were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg) for mesenteric arteries isolation. Porcine coronary arteries (20 to 30 mm from the origin of the proximal portion of left anterior descending arteries) were obtained from a local abattoir.

Reagents and cell lines

Madagascine was supplied by Francesco Epifano and Salvatore Genovese. AMPK-α1+α2 antibodies (ab800039), AMPK-α1 (phospho T183) +α2 (phospho T172) antibodies (ab72845), eNOS antibodies (ab5589), and eNOS (phospho S1177) antibodies (ab184154) were bought from Abcam (Hong Kong) Ltd. (Hong Kong, China). MYPT1 p-MYPT1 (phospho Ser 695) antibodies (sc-33360) were bought from Santa Cruz Biotechnology, Inc (CA, USA). MLC antibodies (3672) and phosphor-MLC (Ser 19) antibodies (3671) were bought form Cell Signaling Technology, Inc (USA). GAPDH (10494-1-AP) antibodies and MYPT1 antibodies (22117-1-AP) were bought form Proteintech Group, Inc (Wuhan, China). SPC was bought from Biomol. Unless otherwise indicated, chemicals were obtained from Sigma-Aldrich (St Louis, USA). The cell HUVECs and HCASMCs were obtained from cell bank of Shanghai Institute (Shanghai, China). The cells used in this study were evaluated before experiment including the expression of eNOS and AMPK in these cell lines. No significant inter-species variations in AMPK and eNOS signaling which affect the results in this study were observed according to previous publications and pre-experiments.

METHODS

Perfusion of rat mesenteric resistance arteries

The rat MRAs were isolated and prepared for perfusion according to the methods by Sun et al (Sun *et al.*, 2009). The MRAs were placed in organ bath maintained at 37 °C, perfused with a modified Krebs solution (modified Krebs solution in mM: NaCl 119.0, KCl 4.7, CaCl2 2.4, MgSO4 1.2, NaHCO3 25.0, KH2PO4 1.2, EDTA 0.03 and D-glucose 11.1 (pH 7.4)) at a constant flow rate of 5 mL/min with a peristaltic pump (Chengdu TME Technology Co, Ltd, China). Changes in the perfusion pressure were recorded by BL-420F biological system (Chengdu TME Technology Co, Ltd, China) (Chen *et al.*, 2015). The endothelium of the MRAs was removed through the perfusion of 1.80 mg/mL sodium deoxycholate in saline for 30 s as described previously (Shiraki *et al.*, 2000). The MRAs were perfused with 100 mM papaverine (PPV) to induce complete relaxation for confirmation of the vascular activity at the end of each experiment.

Simultaneous measurement of $[Ca^{2+}]i$ and force of porcine VSM in Situ

Porcine coronary arteries were placed in 4 °C physiological salt solution (PSS; in mM: 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 $KH2PO_4$, 1.2 $MgCl_2$, 1.25 $CaCl_2$, and 11.5 D-glucose). The arteries were cut into strips (1*4 mm) without endothelium and adventitia. These strips were mounted vertically at the organ bath filled with PSS, gassed with 5% CO_2/95% O_2, and maintained at 37°C. The isometric force of VSM strips was measured by a force transducer (TB-612T, Nihon Koden). Effect of madagascine on contractile force was investigated at the maximum and steady state of SPC or 40 mM K^+ induced constriction.

The contractile force and changes in intracellular Ca^{2+} ($[Ca^{2+}]i$) were simultaneously measured using porcine VSM strips (Fumiaki *et al.*, 2002).The VSM strips were loaded with 12.5 μM fura-2/AM. Changes in $[Ca^{2+}]i$ were continuously recorded with a spectrofluorometer (CAM-230, Japan Spectroscopic) equipped with a randomized optical fiber system (Noriyasu *et al.*, 2006; Todoroki-Ikeda *et al.*, 2000).

Western blot analysis

Total protein was isolated from HUVECs or HCASMCs. The blots on nitrocellulose filter

membrane were probed with corresponding antibodies. The bands were detected and quantified using MultiSpectral imaging system (UVP, Cambridge, UK).

Cell transfection

The HUVECs or HCASMCs cells were transfected with Lipofectamine 2000 (Invitrogen) and AMPK-α1/α2-targeted or a control small interfering RNA (siRNA) oligos (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions (Takara Biotechnology (Dalian) CO., LTD). The siRNA sequence was as follows: 50-ACC GAG CUA UGA AGC AGC UGG GUU U-30.The efficiency of gene silencing was confirmed by Western blotting analysis.

Statistical analysis

Data analysis was conducted in a blinded manner according to single-blind study design. The One-Way ANOVA was used where three or more groups of data were compared. Data were expressed as the mean ± SD. The data followed a normal distribution and each group had equal variances. To further evaluate the data, Kruskal-Wallis rank sum test was used. All experiments were repeated for at least six times.

RESULTS

Effects of madagascine on the vasoconstriction of MRAs

Based on pre-experiments, 0.3-100 μM madagascine were selected to study its vasodilatory effects. Madagascine reduced constriction of rat isolated MRAs induced by 40 mM K^+ in a concentration range of 0.3-100 μM. Madagascine-induced vasodilatation was transient and disappeared within about 6 min. Equal volume of vehicle DMSO did not show any inhibitory effect on the constriction of MRAs (Figure 2). Madagascine also relaxed MRAs pre-contracted by methoxamine and endothelin-1. However, madagascine did not significantly change the basal tension (Figure 3). The rat MRAs remained in good condition after madagascine treatment by washing.

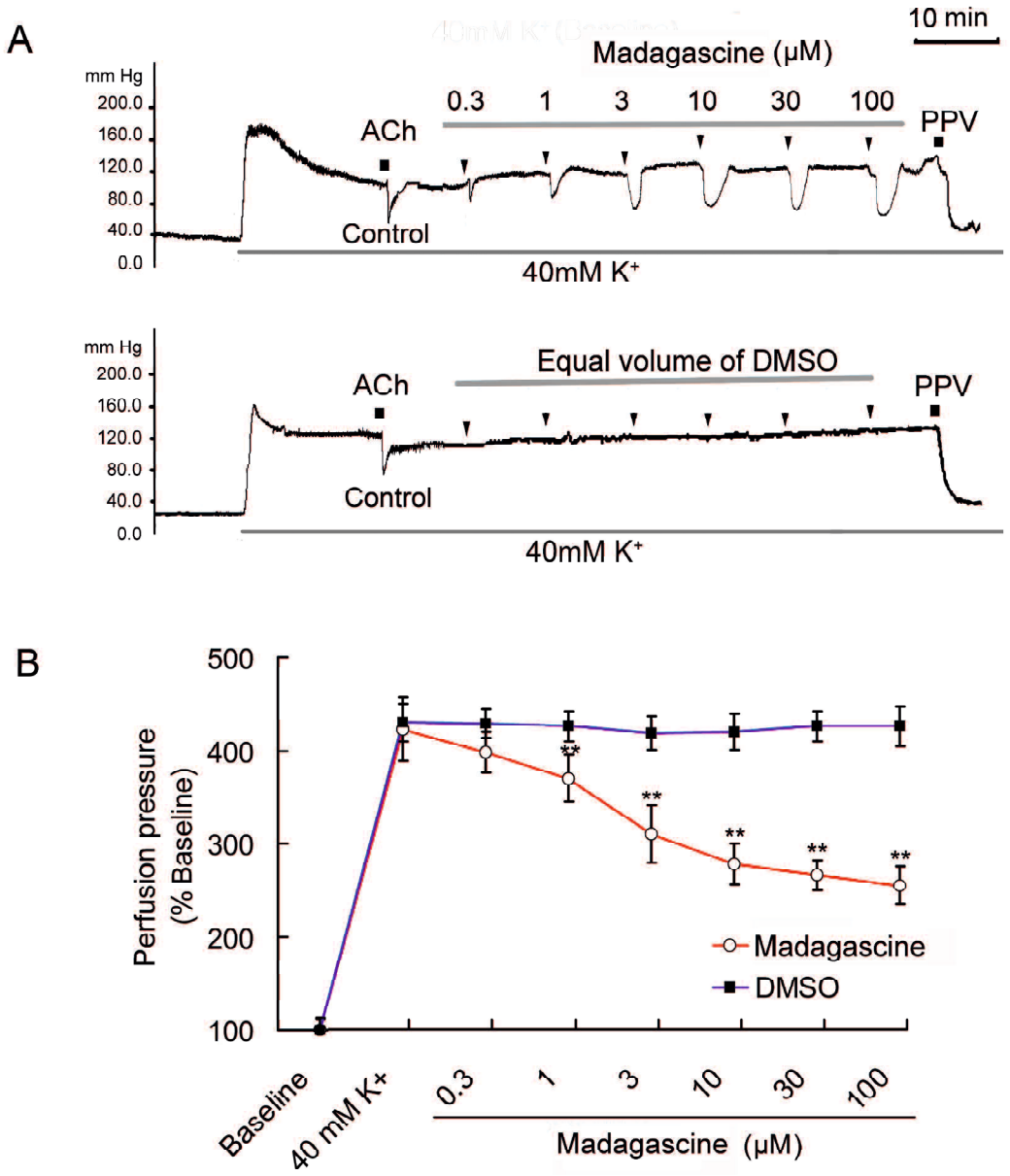

Figure 2. Madagascine (0.3-100 μM) inhibited the constriction of rat mesenteric resistance arteries (MRAs) induced by 40 mM K^+. The doses of madagascine were selected according to pre-experiments. Isolated rat MRAs with intact endothelium were perfused with Krebs solution and the perfusion pressure was stimulated by continuous perfusion of 40 mM K^+. After the elevated perfusion pressure stabilized, Krebs solution containing 40 mM K^+ and madagascine at a concentration of 0.3, 1, 3, 10, 30 or 100 μM was perfused respectively. Acetylcholine (ACh, 1 nM) treatment is a bolus injection. Vasodilatation response to ACh confirms the intact of endothelium. Data are expressed as the mean ± SD and the response to Krebs solution was set as baseline. Other data are the relative values compared with baseline. $^{**}p < 0.01$ compared with 40 mM K^+,

n=6 tissues.

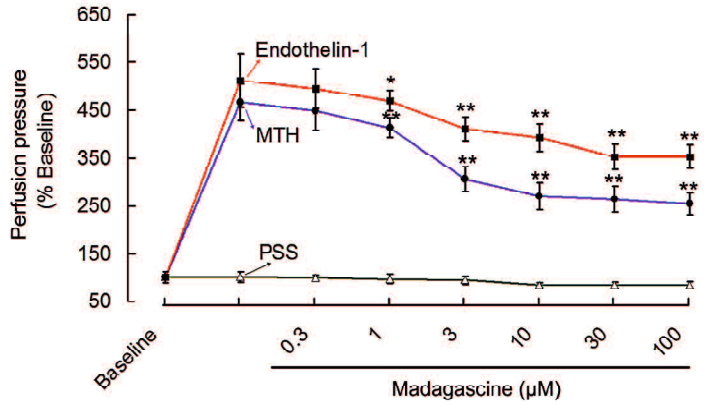

Figure 3. Madagascine (0.3-100 μM) inhibited the constriction of rat mesenteric resistance arteries (MRAs) induced by endothelin-1 (10 nM) and methoxamine (MTH, 7 μM). The perfusion pressure of isolated rat MRAs with intact endothelium were stimulated by continuous perfusion of endothelin-1/methoxamine respectively. After the elevated perfusion pressure stabilized, Krebs solution containing endothelin-1/methoxamine and madagascine at a concentration of 0.3, 1, 3, 10, 30 or 100 μM was perfused respectively. The intact of endothelium was confirmed through vasodilatation response to ACh. Data are expressed as the mean ± SD and the response to Krebs solution was set as baseline. Other data are the relative values compared with baseline. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with endothelin-1/methoxamine, n=6 tissues.

Effects of L-NAME and compound C on madagascine induced vasodilatation

Madagascine did not induced vasodilatation on 40 mM K^{+}-induced constriction of rat MRAs without epithelium (Figure 4). In MRAs with an intact endothelium, madagascine exerted vasodilatation effect was significantly blocked by NO synthase inhibitor L-NAME and AMPK inhibitor compound C respectively. These results suggest that AMPK/eNOS signaling pathway is involved in madagascine-induced vasodilatation (Figure 4). Both AMPK/eNOS and AMPK/AKT/eNOS are known to be related to increase the production of NO (Bradley *et al.*, 2010; Wei *et al.*, 2016). As shown in figure 5, madagascine-induced vasodilatation in the presence of SC66 was more transient than that in the absence of SC66. However, in this study, AKT inhibitor did not significantly block madagascine induced vasodilatation rate (Figure 5). The potential role of AMPK/eNOS pathway was mainly studied in this study.

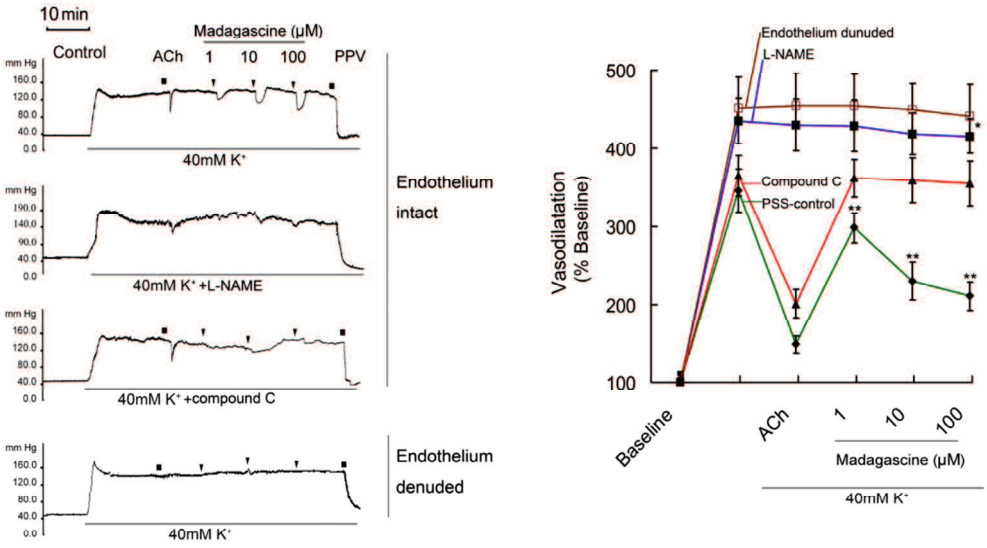

Figure 4. Effects of compound C and L-NAME on madagascine-exerted vasodilatation. After 40 mM K^+ induced high perfusion pressure stabilized, Krebs solution containing 40 mM K^+ + L-NAME (100 μM) and 40 mM K^+ + compound C (20 μM) was perfused respectively. And after the perfusion pressure stabilized, Krebs solution containing 40 mM K^++L-NAME (100 μM) + madagascine and 40 mM K^+ + compound C (20 μM) + madagascine was perfused. Acetylcholine (ACh, 1 nM) treatment is a bolus injection. Vasodilatation response to ACh confirms the intact of endothelium. Data are expressed as the mean ± SD and the response to Krebs solution was set as baseline. Other data are the relative values compared with baseline. ∗p < 0.05 compared with stable elevated perfusion pressure by Krebs solution containing both 40 mM K+ and L-NAME, n = 6 tissues; ∗∗p < 0.01 compared with stable elevated perfusion pressure by Krebs solution containing 40 mM K+ (PSS control), n = 6 tissues.

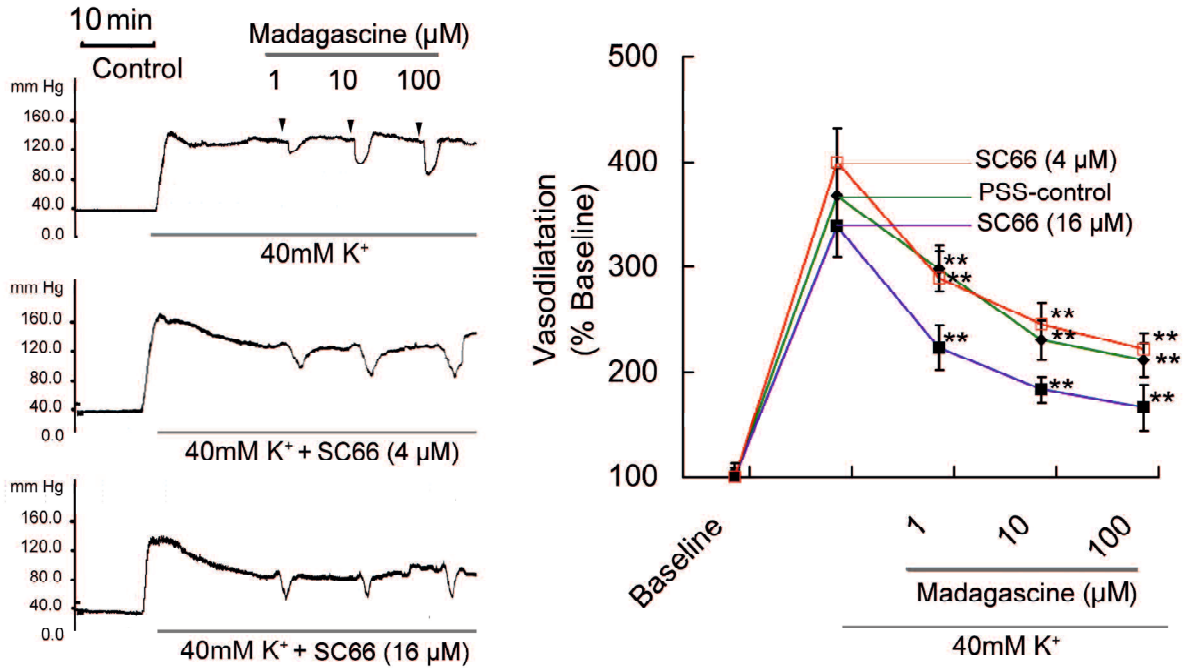

Figure 5. Effects of AKT inhibitor SC66 on madagascine-exerted vasodilatation. After 40 mM K^+ induced high perfusion pressure stabilized, Krebs solution containing 40 mM K^+ + SC66 (4 μM) and 40 mM K^+ + SC66 (16 μM) was perfused respectively. And after the perfusion pressure stabilized, Krebs solution containing 40 mM K^++ SC66 (4 μM) + madagascine and 40 mM K^+ + SC66 (16 μM) + madagascine was perfused. Data are expressed as the mean ± SD and the response to Krebs solution was set as baseline. Other data are the relative values compared with baseline. $^{**}p < 0.01$ compared with stable elevated perfusion pressure by Krebs solution containing 40 mM K^+ and SC66, n=6 tissues.

Effects of madagascine on AMPK and eNOS in HUVECs

To validate the involvement of AMPK/eNOS signaling pathway in madagascine exerted vasodilatation, the effects of madagascine on AMPK and eNOS were studied using HUVECs. Madagascine, in a concentration range of 0.3-30 μM, induced the phosphorylation of eNOS and the phosphorylation of AMPK respectively in a time dependent manner from 1 - 24 min (Figure 6). The effects of AMPK knockdown by siRNA in HUVECs were confirmed by Western blotting analysis (Figure 7A and B). After siRNA knockdown of AMPK, the madagascine-induced phosphorylation of eNOS was abrogated, indicating that madagascine-induced phosphorylation of AMPK activated the phosphorylation of eNOS, leading to nitrite-mediated blood vessel relaxation (figure 7).

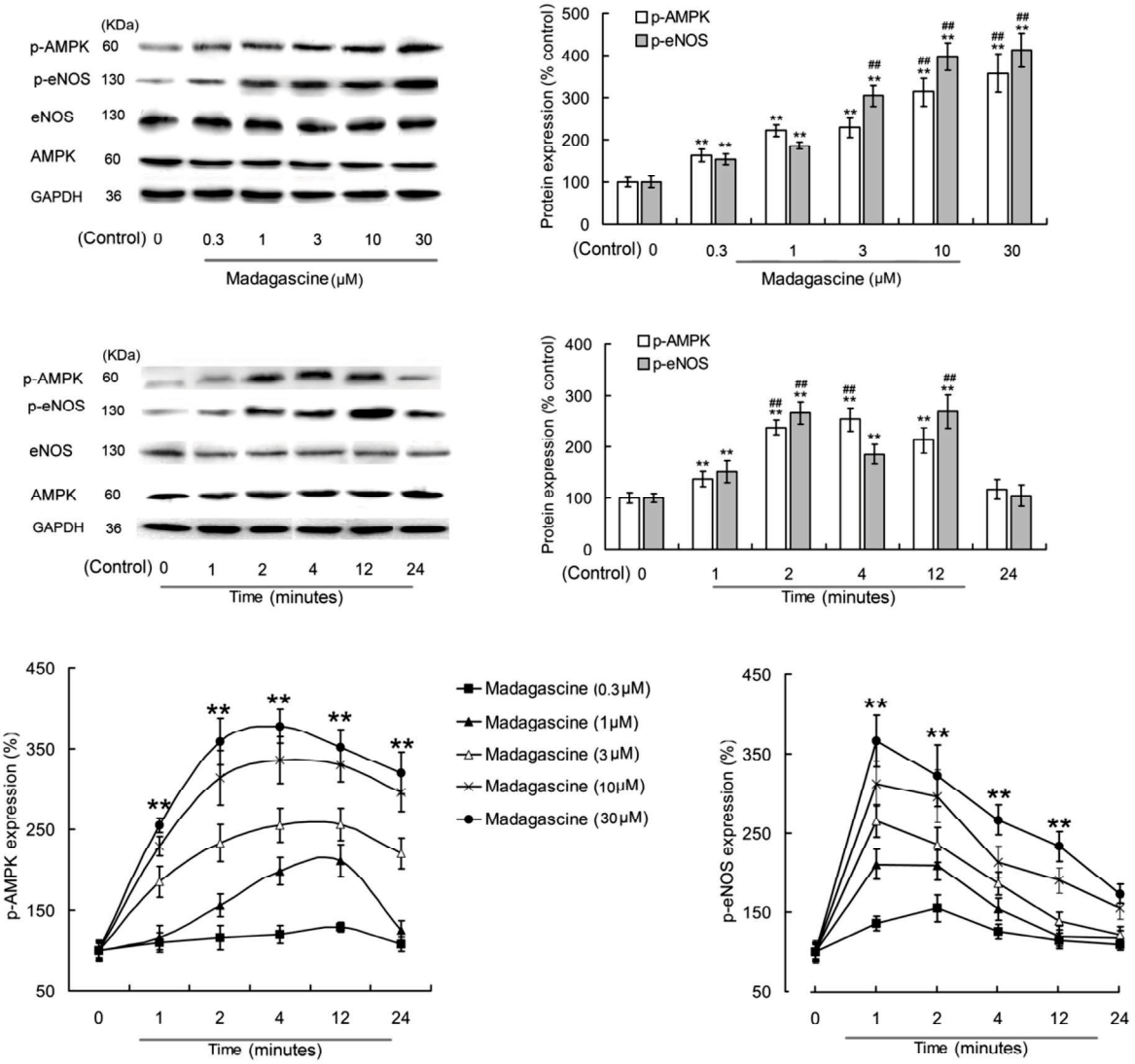

Figure 6. Effects of madagascine on AMPK phosphorylation in human umbilical vein endothelial cells. (A) 0.3-30 μM madagascine increased the phosphorylation of eNOS and the phosphorylation of AMPK with incubation time of 4 minutes. (B) 10 μM madagascine increased the phosphorylation of eNOS and the phosphorylation of AMPK with incubation time of 1-24 minutes. (C) Madagascine in different concentrations increased the phosphorylation of eNOS and the phosphorylation of AMPK with different incubation times. Data are expressed as the mean ± SD and the data. The group without madagascine incubation is set to a relative value of 100% (control). Other data are the relative values compared with control. **$p < 0.01$ compared with the control, n=6 tissues; $^{\#\#}$ $p < 0.01$ compared with the corresponding data in 0.3 μM madagascine treated group or 1 min treated group.

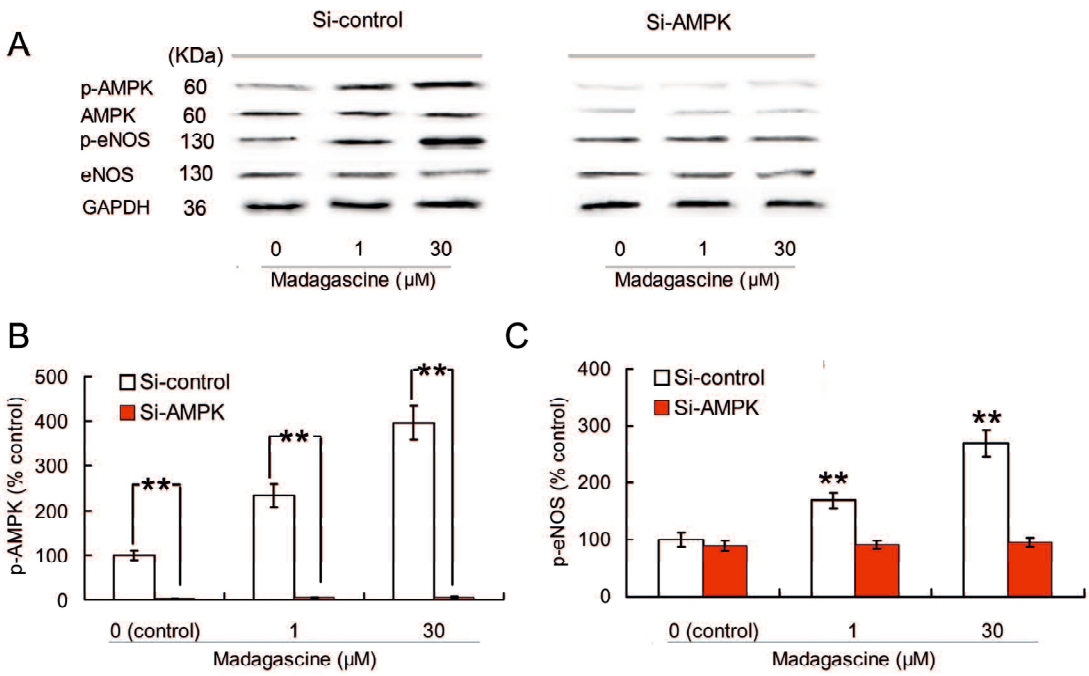

Figure 7. Effects of small interfering RNA knockdown of AMPK on madagascine induced phosphorylation of eNOS (p-eNOS) in human umbilical vein endothelial cells. Data are expressed as the mean ± SD and the data observed from madagascine untreated si-control group is set to a relative control of 100%. Other data are the relative values compared with control. $^{**}p < 0.01$ compared with control or as indicated, n=5 samples.

Effects of madagascine on SPC induced abnormal constriction

Madagascine did not affect 40 mM K^+-induced constriction in rat MRAs without epithelium,, suggesting that madagascine-activated AMPK induced vasodilatation of VSM is Ca^{2+}-independent, because K^+ depolarization induced vasoconstriction is a typical Ca^{2+} dependent constriction (Satoshi *et al.*, 2002; Somlyo, 2002).

As SPC is found to induce Ca^{2+} independent vasoconstriction through activation of ROCK which is involved in hypertension and vasospasm (Satoshi *et al.*, 2002), the effects of madagascine on vasospasm induced by SPC were investigated. The porcine coronary arteries were used here because porcine coronary arteries are cholesterol-enriched and cholesterol potentiates the Ca^{2+} independent of VSM constriction mediated by SPC as described above. As shown in figure 8A, madagascine affects neither the increase of $[Ca^{2+}]i$ nor the constriction of VSM induced by 40 mM K^+. However, SPC induced VSM constriction was significantly reduced by madagascine without significant affecting the slightly elevated $[Ca^{2+}]i$ (Figure 8B); and

madagascine induced vasodilatation was abolished by AMPK inhibitor compound C (Figure 8C), indicating that madagascine reduced SPC-induced Ca^{2+} independent vasoconstriction via activating AMPK.

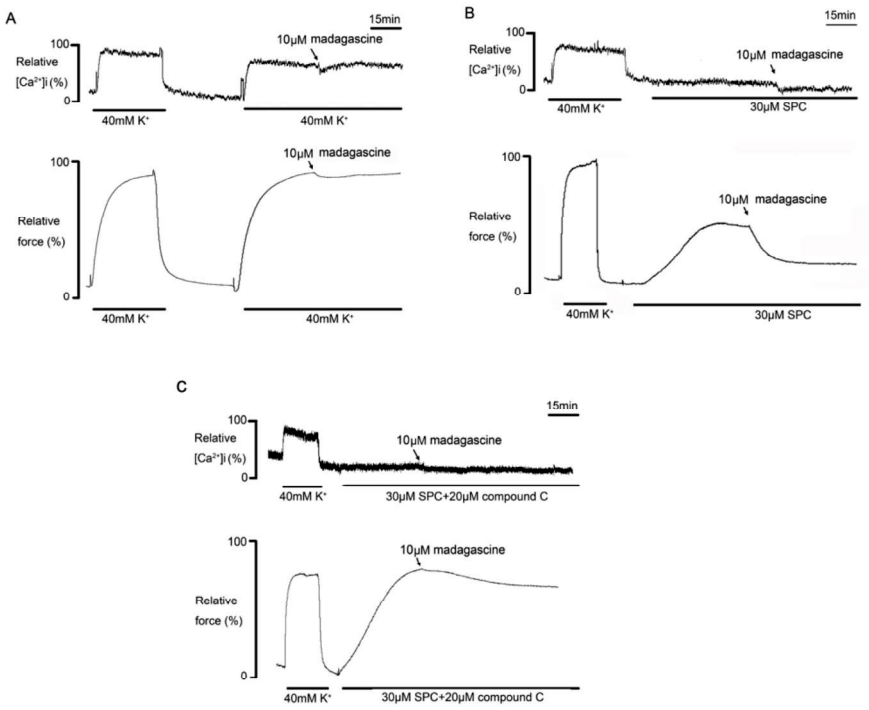

Figure 8. Effects of madagascine on $[Ca^{2+}]i$ and force of porcine vascular smooth muscle constriction induced by sphingosylphosphorylcholine (SPC). Force and fluorescence ratios were expressed as a percentage and the response to 40 mM K^+ was set to 100%. Every experiment was repeated at least 5 times.

Effects of madagascine on AMPK, MYPT1 and MLC20 in HCASMCs

To validate the involvement of AMPK in madagascine-reduced abnormal constriction induced by SPC, the effects of madagascine on the phosphorylation of MYPT1 and phosphorylation of MLC_{20} in VSM cells with siRNA knockdown of AMPK were studied. The effects of AMPK knockdown by siRNA in HCASMCs were confirmed by Western blotting analysis (Figure 9A and C). The phosphorylation of MYPT1 and the phosphorylation of MLC_{20} were significantly increased in HCASMCs incubated with SPC (Figure 9A, E and F). Madagascine significantly decreased both SPC-elevated phosphorylation of MYPT1 and the phosphorylation of MLC_{20}. The inhibitory effects of madagascine on the phosphorylation of MYPT1 and the phosphorylation of MLC_{20} were abrogated by siRNA knockdown of AMPK (figure 9A, E and F), indicating that

madagascine-induced phosphorylation of AMPK inhibited the activity of ROCK (the expression of phosphorylate MYPT1 represents the ROCK activity), leading to the vasodilatation of Ca^{2+}-independent vasoconstriction. In order to prevent the false-positive results by ROCK independent phosphorylation of MYPT1(Kaplun *et al.*, 2003), ROCK inhibitor Y-27632 was also used. As shown in figure 9 B and D, the inhibitory effects of madagascine on the phosphorylation of MYPT1 were also significantly abrogated by Y-27632, which confirms that madagascine induced activation of AMPK leads to the inhibition of ROCK (Chen D, Lv B *et al.*,2016).

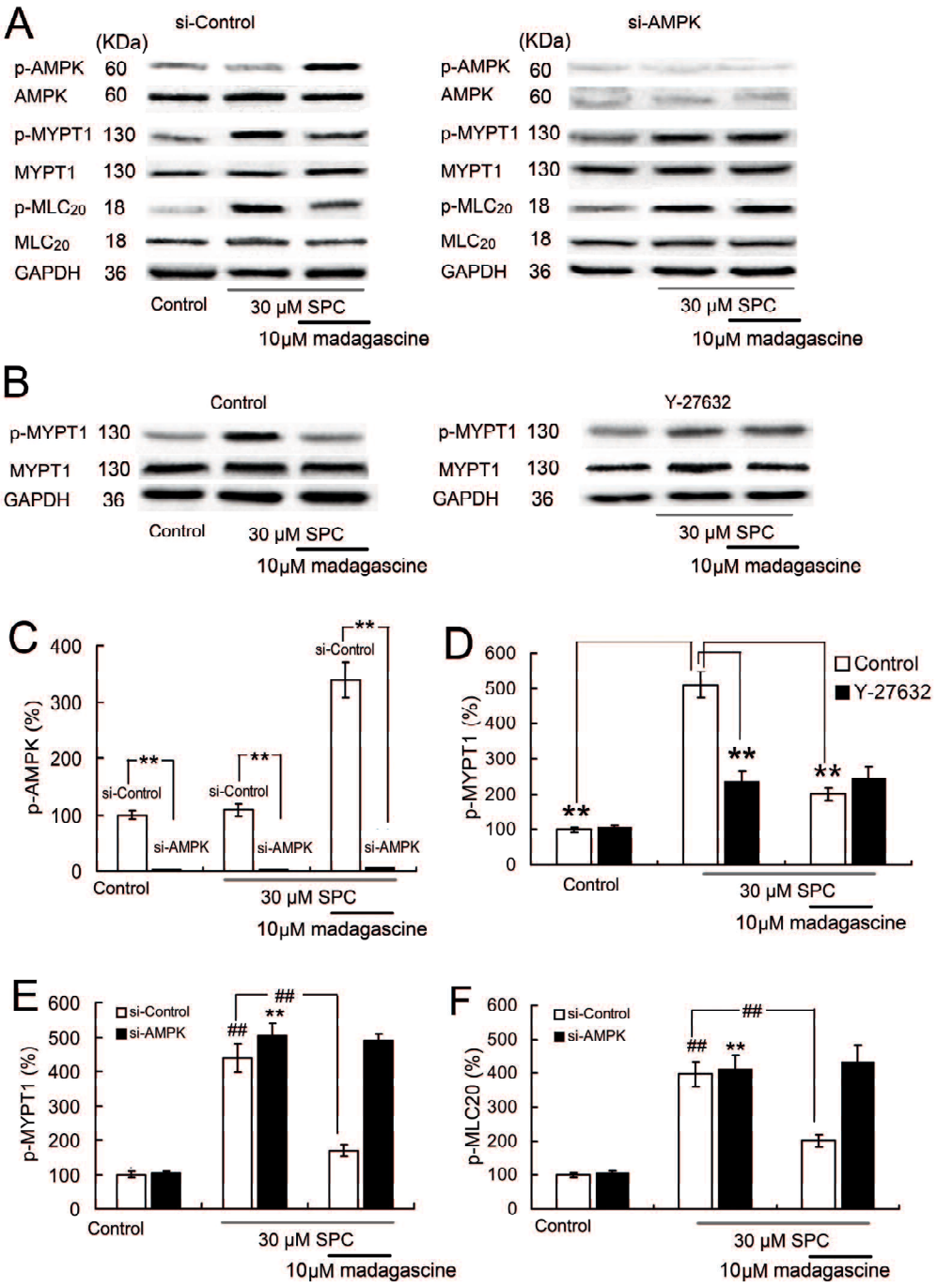

Figure 9. Effects of small interfering RNA knockdown of AMPK and ROCK inhibitor Y27632 (10 μM) on madagascine induced the phosphorylation of MYPT1 and the phosphorylation of MLC_{20} in human coronary artery smooth muscle cells. Data are expressed as the mean ± SD and the data obtained from madagascine untreated si-control group is set to a relative value of 100% (control). Other data are the relative values compared with control. $^{**}p < 0.01$ compared with madagascine untreated si-AMPK group or as indicated, n=6 samples; $^{\#\#}$ $p < 0.01$ compared with data obtained from madagascine untreated si-control group or as indicated, n=6 samples.

DISCUSSION

In this study, madagascine exerted vasodilatory effects in rat MRAs and porcine VSM strips respectively. The phosphorylation of AMPK was significantly increased by madagascine in a concentration and time dependent manner. The phosphorylation of eNOS in HUVECs was significantly increased and the phosphorylation of MYPT1 in HCASMC cells was significantly decreased by madagascine. These results suggest that AMPK-mediated activation of eNOS in epithelium and inhibition of ROCK/MYPT1 in VSM were involved in madagascine-induced vasodilatation (figure 10).

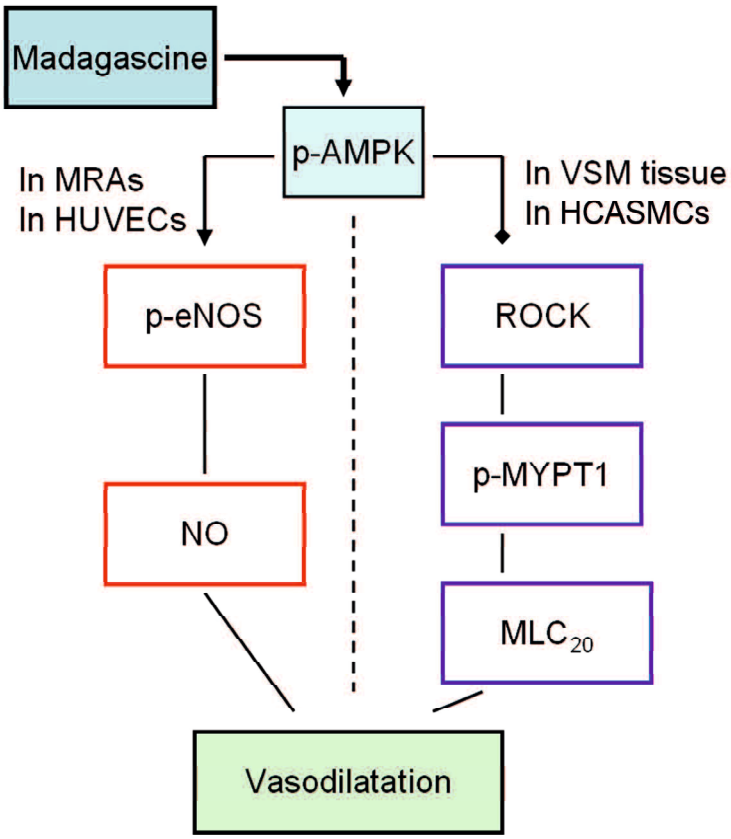

Figure 10. The potential mechanisms involved in madagascine-activated AMPK induced vasodilatation. The solid black squares represent for inhibitory effect; the arrow represents for stimulatory effect. MRAs, mesenteric resistance arteries; VSM, vascular smooth muscle; HUVECs, human umbilical vein endothelial cells; HCASMCs, human coronary artery smooth muscle cells.

In the vasculature, the activation of endothelial AMPK has been shown to phosphorylate eNOS at Ser^{1177} and Ser^{633}, stimulating NO release and subsequent vasodilatation of blood

vessels (Majithiya *et al.*, 2006). Purified AMPK is also reported to phosphorylate eNOS at Thr^{495} in vitro (Mariman *et al.*, 2010). In this study, we also found that in the presence of AKT inhibitor SC66, madagascine induced vasodilatation was more transient than that in the absence of SC66. This suggests that the AMPK/AKT/eNOS pathway may also be involved in madagascine induced vasodilatation. AKT pathway may attribute to increase madagascine induced vasodilatation time. However, how can AKT modulate madagascine induced madagascine needs further study.

In this study, madagascine inhibited 40 mM K^+-induced MRAs constriction and the inhibition was blocked by AMPK inhibitor compound C and eNOS inhibitor L-NAME. These results suggested that AMPK activation in epithelium is involved in madagascine-induced MRAs vasodilatation.

AMPK-mediated relaxation of VSM constriction is also observed where the endothelium is damaged (Majithiya *et al.*, 2006), suggesting that AMPK activation mediated vasodilatation is not dependent on endothelium. AMPK activation can lead to Ca^{2+} independent vasoconstriction (Shuangxi *et al.*, 2011; Somlyo, 2002) and this effect was also confirmed in the present study as madagascine did not show any relaxant effects on the constriction of MRAs without endothelium in the presence of 40 mM K^+ (data not shown). Madagascine significantly inhibited the VSM constriction induced by SPC and the inhibition was abolished by compound C, suggesting that AMPK activation is involved in madagascine induced VSM relaxation. In HCASMC cells, SPC-induced increase in the phosphorylation of MYPT1 and the phosphorylation of MLC_{20} were significantly reversed by madagascine. AMPK knock down block madagascine-induced the inhibition of the phosphorylation of MYPT1 (represents for the ROCK activity) and the phosphorylation of MLC_{20}. The results suggest that madagascine-induced AMPK activation is beneficial for amelioration of vasospasm as SPC-mediated activation of ROCK is found to be involved in pathogenesis of vasospasm (Fumiaki *et al.*, 2002; Somlyo, 2002). Although it is applicable to test the drug effects on AMPK or eNOS using HUVEC and HCASMCs (Fumiaki *et al.*, 2002; Noriyasu *et al.*, 2006; Qian *et al.*, 2012; Satoshi *et al.*, 2002; Yang *et al.*, 2009), it is better to use primary cells to study. Due to limited conditions, these experiments will also be carried out in future study.

This study studied the effects of madagascine on both endothelium and VSM because endothelium and VSM play different pathophysiological roles in vascular diseases (Santulli *et al.*,

2011; Santulli *et al.*, 2014). For example, the lack of discrimination between proliferating VSM cells and endothelial cells may increase the risk of late thrombosis following angioplasty(Santulli *et al.*, 2014). Madagascine-induced vasodilatation was transient and this transient vasodilatation effect on blood pressure in vivo needs further study. The highest concentration of madagascine used in this study is 100 μM and it was shown that madagascine and its parent compound emodin shows almost no cell toxicity on normal cells but cancer cells (Epifano et al., 2013; Wei et al., 2013). The rat MRAs kept good condition after madagascine treatment by washing in this study, which also confirmed almost no cell toxicity of madagascine. However, the higher concentration of madagascine is also to be used to test its effects *in vivo* study. The aim of this study is to study the vasodilatory effect of madagascine on vasoconstriction *in vitro,* and to uncover weather AMPK activation is involved in madagascine induced vasodilatation. The other roles including potassium channel, alpha adrenergic receptors, and some vasodilatation factors in madagascine induced vasodilatation maybe respectively studied in future.

Cardiovascular diseases are often associated with co-occurring atherosclerosis, hypercholesterolemia, and inflammation, and madagascine induced activation of AMPK also is potentially beneficial for the prevention against and amelioration of the co-occurring disorders based on its modulation on VSM and circadian rhythm (Fan *et al.*, 2011). Madagascine has potent biological activity and is safer as a potent AMPK activator. Madagascine-induced activation of AMPK leads to inhibition of vasoconstriction suggests its potential value in amelioration of vasospasm related cardiovascular diseases.

AUTHOR CONTRIBUTIONS

Designed research: Y.L, F.E and D.P.C. Performed research: D.P.C, B.C.L, Y.J.X, S.S.H, S.G., and D.D.Y. Contributed reagents or analytic tools: Y.L, F.E, S.G, and S.K. Analyzed data: B.C.L and Y.J.X; Wrote the paper: Y.L and D.P.C.

ACKNOWLEDGEMENT

This study was supported by the National Natural Science Foundation of China (grant No. 30772601 and 81600440). The authors wish to thank Pengyuan Sun and Lei Shi for their helpful discussions and reading of this manuscript.

REFERENCES

Bradley EA, Eringa ECStehouwer CD, Korstjens I, Gp VNA, Musters R, Sipkema P, *et al.* (2010). Activation of AMP-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside in the muscle microcirculation increases nitric oxide synthesis and microvascular perfusion. *Arteriosclerosis Thrombosis & Vascular Biology* **30**(6)**:** 1137-1142.

Chen D, Xiong Y, Lin Y, Tang Z, Wang J, Wang L, *et al.* (2015). Capsaicin alleviates abnormal intestinal motility through regulation of enteric motor neurons and MLCK activity: Relevance to intestinal motility disorders. *Molecular Nutrition & Food Research* **59**.

Cox RH, Rusch NJ (2002). New expression profiles of voltage-gated ion channels in arteries exposed to high blood pressure. *Microcirculation* **9**(4)**:** 243-257.

Delle Monache G, Delle Monache F, Di Benedetto R, Oguakwa JU (1987). New metabolites from Psorospermum tenuifolium. *Phytochemistry* **26**(9)**:** 2611-2613.

Ee GCL, Sukari M, Aspollah M, Sim W, Lim C (2011). Cratoxylum glaucum and cratoxylum arborescens (Guttiferae)-two potential source of antioxidant agents. *Asian Journal of Chemistry* **23**(2)**:** 569-572.

Epifano F, Fiorito S, Carlucci G, Locatelli M, Genovese S (2013). Phytochemistry and pharmacognosy of naturally occurring prenyloxyanthraquinones. *Curr Drug Targets* **14**(9)**:** 959-963.

Fan W, Downes M, Atkins A, Yu R, Evans RM (2011). Nuclear receptors and AMPK: resetting metabolism. *Cold Spring Harb Symp Quant Biol* **76**.

Nakao F, Kobayashi S, Mogami K, Mizukami Y, Shirao S, Miwa S, *et al.* (2002). Involvement of Src family protein tyrosine kinases in Ca(2+) sensitization of coronary artery contraction mediated by a sphingosylphosphorylcholine-Rho-kinase pathway. *Circulation Research* **91**(10)**:** 953-960.

Hisham NF, Bayraktutan U (2012). Epidemiology, Pathophysiology, and Treatment of Hypertension in Ischaemic Stroke Patients. *Journal of Stroke & Cerebrovascular Diseases the Official Journal of National Stroke Association* **22**(7)**:** e4–e14.

Iinuma M, Tosa H, Ito T, Tanaka T, Aqil M (1995). Two prenylated anthrones in Harungana madagascariensis. *Phytochemistry* **40**(1)**:** 267-270.

Ji L (2013). Association between Polymorphisms in the Renin-Angiotensin-Aldosterone System Genes and Essential Hypertension in the Han Chinese Population. *Plos One* **8**(8)**:** 618-618.

Kaplun L, Ivantsiv Y, Bakhrat A, Raveh D (2003). DNA damage response-mediated degradation of Ho endonuclease via the ubiquitin system involves its nuclear export. *Journal of Biological Chemistry* **278**(49)**:** 48727-48734.

Kondo K, Okuno T, Konishi K, Saruta T, Kato E (1979). Central and peripheral effects of bradykinin and

prostaglandin E2 on blood pressure in conscious rats. *Naunyn-Schmiedeberg's archives of pharmacology* **308**(2)**:** 111-115.

Kumar P, Mastan K, Chowdhary R, Shanmugam K (2012). Oral manifestations in hypertensive patients: A clinical study. *Journal of Oral & Maxillofacial Pathology* **16**(2)**:** 215-221.

Machino T, Murakoshi N, Sato A, Xu DZ, Hoshi T, Kimura T*, et al.* (2014). Anti-hypertensive effect of radiofrequency renal denervation in spontaneously hypertensive rats ☆. *Life sciences* **110**(2)**:** 86-92.

Majithiya JB, Balaraman R (2006). Metformin reduces blood pressure and restores endothelial function in aorta of streptozotocin-induced diabetic rats. *Life sciences* **78**(22)**:** 2615-2624.

Mariman ECM, Wang P (2010). Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cellular & Molecular Life Sciences* **67**(8)**:** 1277-1292.

Mbaveng AT, Kuete V, Nguemeving JR, Beng VP, Nkengfack AE, Meyer JM*, et al.* (2008). Antimicrobial activity of the extracts and compounds obtained from Vismia guineensis (Guttiferae). *Asian J Trad Med* **3:** 211-223.

Morikage N, Kishi H, Sato M, Guo F, Shirao S, Yano T*, et al.* (2006). Cholesterol primes vascular smooth muscle to induce Ca2 sensitization mediated by a sphingosylphosphorylcholine-Rho-kinase pathway: possible role for membrane raft. Circulation Research 99(3): 299-306.

Qian XU, Cao K, Zhou XH, Wang XJ, Wang YF, Chao DU (2012). Effects of paeonol on expression of eNOS and level of NO in HUVECs injured by homocysteine. *Chinese Traditional Patent Medicine*.

Reihill JA, Ewart MA, Hardie DG, Salt IP (2007). AMP-activated protein kinase mediates VEGF-stimulated endothelial NO production. *Biochemical & Biophysical Research Communications* **354**(4)**:** 1084-1088.

Ritchie E, Taylor W (1964). The constituents of HarunganaMadagascariensis Poir. *Tetrahedron Letters* **5**(23)**:** 1431-1436.

Salihi D (2013). Endothelium derived relaxation factors reduce sulfur dioxide-induced aortic relaxation. *Open Journal of Molecular & Integrative Physiology* **3**(4)**:** 181-185.

Santulli G, Basilicata MF, De SM, Del GC, Anastasio A, Sorriento D*, et al.* (2011). Evaluation of the anti-angiogenic properties of the new selective αVβ3 integrin antagonist RGDechiHCit. *Journal of Translational Medicine* **9**(1)**:** 1-10.

Santulli G, Wronska A, Uryu K, Diacovo TG, Gao M, Marx SO*, et al.* (2014). A selective microRNA-based strategy inhibits restenosis while preserving endothelial function. *Journal of Clinical Investigation* **124**(9)**:** 4102-4114.

Shirao S, Kashiwaqi S, Sato M, Miwa S, Nakao F, Kurokawa T*, et al.* (2002). Sphingosylphosphorylcholine is a novel messenger for Rho-kinase-mediated Ca2+ sensitization in the bovine cerebral artery: unimportant role for protein kinase C. Circulation Research 91(2): 112-119.

Shiraki H, Kawasaki H, Tezuka S, Nakatsuma A, Kurosaki Y (2000). Endogenous calcitonin gene-related peptide (CGRP) mediates adrenergic-dependent vasodilation induced by nicotine in mesenteric resistance arteries of the rat. *British journal of pharmacology* **130**(5)**:** 1083-1091.

Shuangxi W, Bin L, Benoit V, Ming-Hui Z (2011). Inhibition of the AMP-activated protein kinase-α2 accentuates agonist-induced vascular smooth muscle contraction and high blood pressure in mice. *Hypertension* **57**(5)**:** 1010-1017.

Somlyo AP, Somlyo AV (1994). Signal transduction and regulation in smooth muscle. *Nature* **372**(6503)**:** 231-236.

Somlyo AV (2002). New roads leading to Ca2+ sensitization. *Circulation Research* **91**(2)**:** 83-84.

Song P, Kim JH, Ghim J, Yoon JH, Lee A, Kwon Y*, et al.* (2013). Emodin regulates glucose utilization by activating AMP-activated protein kinase. *J Biol Chem* **288**(8)**:** 5732-5742.

Stahmann N, Woods A, Carling D, Heller R (2006). Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca2+/calmodulin-dependent protein kinase kinase beta. *Mol Cell Biol* **26**(16)**:** 5933-5945.

Subramaniam A, Shanmugam MK, Ong TH, Li F, Perumal E, Chen L*, et al.* (2013). Emodin inhibits growth and induces apoptosis in an orthotopic hepatocellular carcinoma model by blocking activation of STAT3. *Br J Pharmacol* **170**(4)**:** 807-821.

Sun P, Jin X, Koyama T, Li S, Kitamura Y, Kawasaki H (2009). R-(-)-alpha-methylhistamine, a histamine H3 receptor agonist, induces endothelium-dependent vasodilation in rat mesenteric resistance arteries. *Biological & pharmaceutical bulletin* **33**(1)**:** 58-63.

Todoroki-Ikeda N, Mizukami Y, Mogami K, Kusuda T, Yamamoto K, Miyake T*, et al.* (2000). Sphingosylphosphorylcholine induces Ca2+ -sensitization of vascular smooth muscle contraction: possible involvement of Rho-kinase. *FEBS letters* **482**(1-2)**:** 85-90.

Wang S, Liang B, Viollet B, Zou MH (2011). Inhibition of the AMP-activated Protein Kinase Alpha 2 Accentuates Agonist-Induced Vascular Smooth Muscle Contraction and High Blood Pressure in Mice. *Hypertension* **57**(5)**:** 1010-1017.

Wei R, Ma S, Wang C, Ke J, Yang J, Li W*, et al.* (2016). Exenatide exerts direct protective effects on endothelial cells through AMPK/Akt/eNOS pathway in a GLP-1 receptor dependent manner. *American Journal of Physiology Endocrinology & Metabolism*.

Wei WT, Lin SZ, Liu DL, Wang ZH (2013). The distinct mechanisms of the antitumor activity of emodin in different types of cancer (Review). *Oncology Reports* **30**(6)**:** 2555-2562.

Yang JH, Wang N, Jing-Yan LI, Zhang JJ, Feng P (2009). Effects of Resveratrol on SIRT1 Expression,eNOS Activity

and NO Secretion of Hyperlipid Cultured HUVEC. *China Pharmacy*.

Yue HW, Jing QC, Liu PP, Liu J, Li WJ, Zhao J (2015). Sphingosylphosphorylcholine in cancer progress. *International Journal of Clinical & Experimental Medicine* **8**(8)**:** 11913-11921.

Chen D, Lv B, Kobayashi S, Xiong Y, Sun P, Lin Y, Genovese S, Epifano F, Hou S, Tang F, Ji Y, Yu D（2016）. Madagascine Induces Vasodilatation via Activation of AMPK. Front Pharmacol **7:** 435.