Songklanakarin J. Sci. Technol.
40 (6), 1248-1258, Nov. – Dec. 2018

http://www.sjst.psu.ac.th

Original Article

Vasorelaxant mechanisms of camboginol from *Garcinia dulcis* in normotensive and 2-kidneys-1-clip hypertensive rat

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Received: 7 April 2017; Revised: 18 July 2017; Accepted: 30 July 2017

Abstract

Endothelial dysfunction along with an increase in reactive oxygen species observed in 2-kidneys-1-clip (2K1C) hypertensive rats escalates hypertension. This study aimed to investigate the vasorelaxant effect of camboginol from *Garcinia dulcis* which is a robust antioxidant in normotensive and 2K1C hypertensive rats (n=6, each). Intravenous injection of camboginol showed a transient reduction in arterial blood pressure and restored both the impaired baroreflex sensitivity and the elevated plasma malondialdehyde observed in the 2K1C rat model. Experiments in isolated thoracic aorta revealed the vasorelaxant action of camboginol with pD_2 of 9.67±0.19 and 8.01±0.66 in normotensive and 2K1C hypertensive rats, respectively. The mechanisms of its actions involved the different extent of endothelial nitric oxide and prostacyclin signaling pathway and opening of the ATP-activated potassium channel. Camboginol also enhanced an endothelial nitric oxide synthase expression in the isolated vessel from 2K1C rats. It is concluded that vasorelaxant mechanisms of camboginol may involve its antioxidant activity.

Keywords: camboginol, *Garcinia dulcis*, 2-kidneys-1-clip, vasorelaxation, antioxidant

1. Introduction

Normal arterial blood pressure is regulated by the balance between vasodilative and vasoconstrictive mechanisms. The possible mechanisms responsible for the development of pathological hypertension are the impairment of endothelial-derived vasodilative production such as nitric oxide (NO) or prostacyclin (PGI_2) in addition to an increase in circulating vasoconstrictors such as angiotensin II (AII). In an animal model, 2-kidneys-1-clip (2K1C) renovascular hypertension (RVH), an impairment of endothelium-dependent vasorelaxation was reported (Choi *et al.*, 2014). Moreover, an increase in plasma AII and an overproduction of reactive oxygen species (ROS), especially superoxide anion (O_2^-), were also observed (McIntyre, Bohr, & Dominiczak, 1999). AII was shown to stimulate O_2^- generation by

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increasing the activity of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in cultured rat vascular smooth muscle cell (Griendling, Minieri, Ollerenshaw, & Alexander, 1994) and in intact aortas of AII-infused hypertensive rat (Rajagopalan *et al.,* 1996). The role of O_2^- is related to endothelial dysfunction by which NO can be scavenged by O_2^- to form peroxynitrite ($ONOO^-$) resulting in reduced NO bioavailability (Rubanyi & Vanhoutte, 1986). In addition, O_2^- was shown to involve diminishing baroreflex sensitivity (BRS) in 2K1C hypertension rat model (Botelho-Ono *et al.,* 2011; Queiroz *et al.*, 2012).

The scavenging of excessive body free radical using plant compounds in treatment of hypertension has been widely researched (Edwards *et al.,* 2007; Gilani *et al.,* 2000; Lv *et al.,* 2013; Mozafari, Nekooeian, Panjeshahin, & Zare, 2015). Camboginol is an isoprenylated benzophenone that can be isolated from most *Garcinia* spp., including the folk medicinal plant *Garcinia dulcis* Kurz (family Guttiferae). The chemical structure of camboginol was first elucidated in 1980 by Rao, Venkatswamy, and Pendse (1986) (Figure 1). Its wide range of biological activities included anti-ulcer (Das, Bandyopadhyay, Bhattacharjee, & Banerjee, 1997; Vaananen, Meddings, & Wallace, 1991), antioxidant (Hutadilok-Towa tana, Kongkachuay, & Mahabusarakam, 2007; Yamaguchi, Ariga, Yoshimura, & Nakazawa, 2000a, Yamaguchi, Saito, Ariga, Yoshimura, & Nakazawa, 2000b), anti-cancer (Tanaka *et al.,* 2000; Yoshida *et al.,* 2005), anti-inflammation (Hong *et al.,* 2006; Kim *et al.,* 2008; Liao, Sang, Liang, Ho, & Lin, 2004), and anti-HIV (Balasubramanyam *et al.,* 2004; Mantelingu *et al.,* 2007).

The potent antioxidant activity of camboginol has been previously reported. It could scavenge the free radical 1, 1-diphenyl-2-picrylhydrazyl with a potency that was three times greater than α-tocopherol (vitamin E), a well-known lipid-soluble natural antioxidant, in aqueous ethanol solution (Yamaguchi *et al.,* 2000a). It was also able to scavenge both hydrophilic and hydrophobic agents including ROS (Yama guchi *et al.,* 2000b). Camboginol also exhibited strong anti-oxidation effect in both Fe^{2+}-mediated and non-metal induced human low-density lipoprotein oxidations (Hutadilok-Towatana *et al.,* 2007).

According to the development of hypertension which may be strengthened by an increase in body reactive oxygen or nitrogen species, we aimed to investigate the vasorelaxant effects of camboginol by determining its acute action on arterial blood pressure (ABP) and heart rate (HR), and also its action on BRS and plasma malondialdehyde (MDA) level in anesthetized 2K1C hypertensive and sham operative (SO) normotensive rats. The endothelium-dependent vasorelaxant mechanisms of camboginol action which might occur via the activation of NO signaling pathway was studied in isolated thoracic aortic rings of both groups using nitric oxide synthase inhibitor (Nω-Nitro-L-arginine methyl ester, or L-NAME) and endothelial nitric oxide synthase (eNOS) expression using immunohistochemistry. Other vasorelaxant mechanisms including the involvement of PGI_2 signaling pathway, ATP-activated potassium (K_{ATP}) channel and Ca^{2+}-activated potassium (K_{Ca}) channel were also studied using the specific blockers indomethacin, glibenclamide, and tetraethylammonium (TEA), respectively.

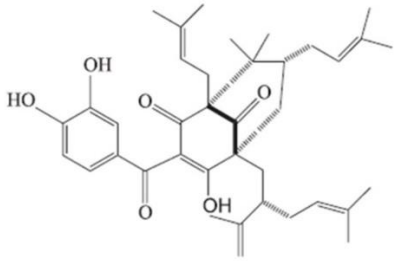

Figure 1. Chemical structure of camboginol which was initially isolated from the latex of *Garcinia cambogia* by Rao *et al.* in 1980 (Molecular formula $C_{38}H_{50}O_6$, Molecular weight 602).

2. Materials and Methods

2.1 Camboginol and chemicals

Camboginol was extracted from the fresh ripe fruits of *Garcinia dulcis* collected in Songkhla Province, Thailand. The voucher specimen was deposited at the herbarium of Prince of Songkla University, Thailand. The extraction procedure of camboginol was described previously (Deacha thai, Mahabusarakam, Phongpaichit, & Taylor, 2005). Its structure is illustrated in Figure 1. Other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA including phenylephrine (PE), sodium nitroprusside (SNP), acetylcholine (ACh), dimethylsulfoxide (DMSO), malondial-dehyde (MDA), thiobarbituric acid (TBA), L-NAME, indomethacin, glibenclamide, and TEA. Pentobarbital sodium was purchased from CEVA Santé Animal, Brussels, Belgium. For the immunohistochemical study, Tissue Freezing Medium® was purchased from Leica, Nussloch, Germany. Rabbit anti-eNOS antibody (Lot # QJ214409), biotinylated goat anti-rabbit IgG (Lot # QE215187), ABC peroxidase staining kit and DAB substrate kit were purchased from Thermo Fisher, Rockford, IL, USA.

2.2 Experimental animals

Male Wistar rats (body weight 150-200 g, n=84) were obtained from the Southern Laboratory Animal Facility (Prince of Songkla University, Thailand). Rats were housed under controlled conditions (temperature 23-24 °C, humidity 50-55%, lighting 0600-1800 h), fed a laboratory diet containing 34.2 mmol sodium chloride/kg dry weight food and were allowed free access to reverse osmosis water.

For the immunohistochemical study of the rat thoracic aorta, the rats (n=7) were purchased from the National Laboratory Animal Center (Mahidol University, Thailand) and housed at the Animal Facility of Department of Zoology, Kasetsart University under controlled conditions as mentioned above. All experiments were approved by the Prince of Songkla University Animal Ethics Committee (Reference No. 31/2014).

2.3 Establishment of 2K1C hypertensive rat and experimental design

Rats were anaesthetized with a single dose of pentobarbital sodium (50 mg/kg body weight [BW] i.p.). Only deeply sedated animals went through the following surgical protocol. The left kidney was exposed through a retro-

peritoneal incision and the left renal artery was exposed and cleared from the surrounding connective tissues. A U-shaped silver clip with a 0.20 mm gap was placed around the left renal artery close to the junction with the abdominal aorta. The muscle and skin layer were sutured separately with catgut and silk No. 4/0, respectively. The SO group included the entire surgery with the exception of renal artery clipping. At the end of the surgery, all animals received a single dose of ampicillin (50 mg/kg BW, i.m.) injection and were allowed to recover in separate cages for 2-3 h under an angle poise lamp and remained untouched for 4 weeks afterward in order to develop hypertension.

The experiments were conducted in two parts: an *in vivo* study and an *in vitro* study. Each part consisted of 4 groups of rats (n=6, each), namely SO + vehicle (SO), SO + camboginol (SO+C), 2K1C + vehicle (2K1C) and 2K1C + camboginol (2K1C+C).

2.4 *In vivo* study of the effect of camboginol on ABP, HR, and BRS

Four weeks after renal artery clipping, the rats were anesthetized with pentobarbital sodium (60 mg/kg BW i.p.; an additional dose was given when necessary) and placed on a thermostatically-controlled heated table to maintain body temperature at 37 ºC. A tracheotomy was performed and the left carotid artery was cannulated, using a polyethylene tube (PE-50) filled with heparinized 0.9% NaCl, and connected to a pressure transducer, coupled to a PowerLab System (ADInstruments, Colorado Springs, CO, USA), to monitor systolic blood pressure (SBP), diastolic blood pressure (DBP), and HR. The right jugular vein was cannulated using PE-50 filled with heparinized 0.9% NaCl. The rats were given 0.9% NaCl solution via jugular vein at a rate of 1.6 mL/h/100g BW until basal ABP and HR were stable. Then, camboginol dissolved in DMSO was given as a bolus injection (0.1 mg/kg BW; volume of injection was 0.05 mL/100g BW) and the same volume of DMSO was given as a vehicle in both SO and 2K1C groups. The maximal response in SBP, DBP, pulse pressure (PP) (SBP-DBP), mean arterial blood pressure (MABP) (DBP+1/3 PP), and HR were then determined.

The effect of camboginol on BRS was performed during the continuous intravenous infusion at the dose of 5 µg/min/kg BW. The experimental dose of camboginol was chosen based on the minimal effective dose of a specific AII receptor antagonist, candesartan (Hiranyachattada, Saetew, & Harris, 2005). After a one-hour equilibration period, the base line values of HR and ABP were recorded. The animals then received acute injections of two sets of vasoactive drugs: PE, a specific α_1 receptor agonist, and SNP, a NO donor, at similar doses of 1, 2, 4, 8, 16, and 32 µg/kg BW. The maximal responses in MABP and HR were then determined. BRS was calculated from $\Delta HR/\Delta MABP$.

At the end of the experiment, blood samples were collected via carotid artery catheter in heparinized tubes and then centrifuged at 4,000 rpm for 10 min. The plasma was then stored at -20 °C until analysis for plasma MDA level. The rats were sacrificed by intravenous injection of saturated magnesium sulfate ($MgSO_4$). Both kidneys were removed, decapsulated, dried on blotting paper, and weighed. The heart was also removed and weighed after carefully clearing away blood vessels, fat, and connective tissues. The organ weight to total body weight ratio was calculated for each organ.

2.5 Determination of plasma MDA

The MDA content was assayed in the form of TBA reacting substances (modified from Ohkawa, Ohishi, & Yagi, 1979). Briefly, the plasma sample, distilled water, and MDA standard were added into tubes followed by 8.1% sodium dodecyl sulfate and 20% acetic acid. The mixture was adjusted to pH 3.5 with NaOH, then 0.8% TBA was added. The mixture was heated in a 95 °C water bath for 60 min and cooled in an ice bath. Distilled water and n-butanol were added and the mixture was then centrifuged. The absorbance of the organic layer was measured using a spectrophotometer. The amount of TBA reactive substances was determined from a standard curve generated by MDA from acid hydrolysis of 1, 1, 3, 3-tetramethoxypropane. The values of plasma MDA were expressed as µmol/L.

2.6 *In vitro* study: Preparation of isolated thoracic aortic rings

Rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and sacrificed by decapitation. The thoracic aorta was dissected and cut into four ring segments approximately 5 mm in length each (two endothelium-intact and two endothelium-denuded rings). The denuded rings were performed by mechanical removal of the endothelium. The rings were mounted in 20-mL organ baths containing 37 °C Krebs Henseleit solution which was composed of (mM) 118.41 NaCl, 4.6 KCl, 1.12 $MgSO_4.7H_2O$, 1.18 KH_2PO_4, 1.9 $CaCl_2$, 25.0 $NaHCO_3$, and 11.66 D-glucose. The pH of the solution was maintained at 7.4 by continuous aeration with 95% O_2 and 5% CO_2. The 1-g resting tension was set and the tension changes during the course of experiment were recorded using a force displacement transducer (Model FT03, Grass Instrument Co., Quincy, MA, USA) connected to a PowerLab System (ADInstruments, USA). The endothelial function of the aortic rings was tested by the addition of 10^{-5} M ACh into the 10^{-7} M PE precontracted rings. A relaxation of 80% was considered acceptable as an intact-endothelium and the disappearance of relaxation was considered as denuded-endothelium (Molina, Hidalgo, & García de Boto, 1992).

2.7 Concentration response curve of camboginol

After a 45-min equilibration period, both endothelium-intact and endothelium-denuded aortic rings from the 2K1C and SO rats were precontracted by addition of 10^{-7} M PE. When the maximal contraction response developed, the tension was recorded. Either camboginol or vehicle (DMSO) was added cumulatively, allowing the final concentration to be 10^{-13}-10^{-5} M and 0.1-0.9%, respectively. Subsequent concentrations were added after the maximal response by the previous concentration developed and recorded.

2.8 Effect of specific inhibitors on vasorelaxation response of camboginol

After a 15-min equilibration period, the endothelium-intact aortic rings from 2K1C and SO rats were incubated with each specific inhibitor for 30 min before precontraction with 10^{-7} M PE. The doses of four inhibitors were 10^{-4} M L-NAME, 10^{-6} M indomethacin, 10^{-5} M glibenclamide, and 10^{-3} M TEA. Then, either camboginol (10^{-13}-10^{-5} M) or vehicle (0.1% DMSO) was added cumulatively after the maximal contraction developed and sustained for 5-7 min. Subsequent concentrations were added after the maximal response from the previously developed and recorded concentration.

2.9 Immunohistochemical study of eNOS expression in thoracic aorta endothelium

One hour after camboginol (0.1 mg/kg + 5 μg/min/kg) was given via the jugular vein of the anesthetized 2K1C rat, it was then terminated by an overdose injection of pentobarbital sodium and the thoracic aorta was removed, dissected, and cut into 5 mm segments. The segments were fixed in 4% buffered formaldehyde, embedded in Tissue Freezing Medium®, and cut on a cryostat. eNOS expression in the aortic sections was evaluated using floating-technique immunohistochemistry. Following several standard pretreatment steps, the sections were incubated with rabbit anti-eNOS antibody (diluted 1:500) at 4 °C for 24 h on a shaker. Next, the sections were incubated with biotinylated goat anti-rabbit IgG (diluted 1:2000) for 1 h, and finally stained using the ABC peroxidase staining kit and metal enhanced DAB substrate kit. The sections were mounted onto slides, covered with a cover slip, and then digitized using an Olympus DP 73 microscope (Olympus Optical Co, Ltd, Tokyo, Japan). For this immunohistochemical study, the animals were divided into 4 groups; control (n=2), negative control (n=2), 2K1C (n=2), and 2K1C+C (n=3).

2.10 Statistical analyses

All data are expressed as mean±S.E.M. The degree of vasorelaxation in each experiment was expressed as percent relaxation from PE (10^{-7} M) precontraction tension. The negative logarithm (pD_2) value was calculated using GraphPad Prism 5 (San Diego, CA, USA). Significant differences between the group means were determined using ANOVA followed by Student-Newman-Keuls post-hoc test or Student t-test. A p value < 0.05 was considered significantly different.

3. Results

3.1 Changes in body weight, kidney weight, and cardiac mass

The body weight changes between the SO and 2K1C rats after four weeks of experimental renal stenosis were not significantly different (Table 1). The left clipped and the right non-clipped kidney weights of the 2K1C rats decreased by 73% and increased by 24% compared to the respective ipsilateral kidneys of the SO rats. The atrophic kidney was due to the reduction in renal blood flow while the right hypertrophic kidney was due to compensation. The cardiac mass of 2K1C groups significantly increased in comparison to the SO groups. This finding suggested the increased afterload in this rat model.

3.2 Acute effect of camboginol on ABP and HR

Four weeks after experimental renal stenosis, the resting SBP, DBP, PP, and MABP in the 2K1C rats were significantly higher than the SO rats (SBP 208±8 vs. 155±4, DBP 143±4 vs. 123±4, PP 64±7 vs. 32±3, and MABP 165±5 vs. 134±4 mm Hg, respectively; P<0.05) (Figure 2). However, the resting HR of the 2K1C rats was not significantly different from the SO rats (212±5 vs. 210±5 bpm).

Table 1. Comparisons of pre-body weight (BW) at the beginning of experiment and post-BW at the end of experiment, left and right kidney weight (KW), and cardiac mass in 2-kidneys-1-clip (2K1C) and sham operated (SO) rats treated with either vehicle (V) or camboginol (C) at 4 weeks after induction of hypertension.

Parameters	SO+V	SO+C	2K1C+V	2K1C+C
Number of rats	6	6	6	6
Pre-BW (g)	206±4	186±14	194±9	183±14
Post-BW (g)	388±3	350±17	379±20	349±10
△BW (g)	182±6	160±21	185±19	161±22
Left KW (g)	1.05±0.02	0.96±0.03	0.28±0.07*	0.24±0.03*
Left KW/BW (%)	0.27±0.01	0.28±0.01	0.07±0.01*	0.08±0.01*
Right KW (g)	1.18±0.02	0.98±0.03	1.46±0.07*	1.30±0.10*
Right KW/BW (%)	0.30±0.01	0.28±0.01	0.39±0.02*	0.36±0.02*
Cardiac mass (g)	1.06±0.02	1.01±0.01	1.23±0.12*	1.17±0.03*
Cardiac mass/BW (%)	0.27±0.01	0.29±0.01	0.32±0.02*	0.34±0.01*

Data are mean±S.E.M.
*P<0.05 compared with respective SO groups (Student t-test).

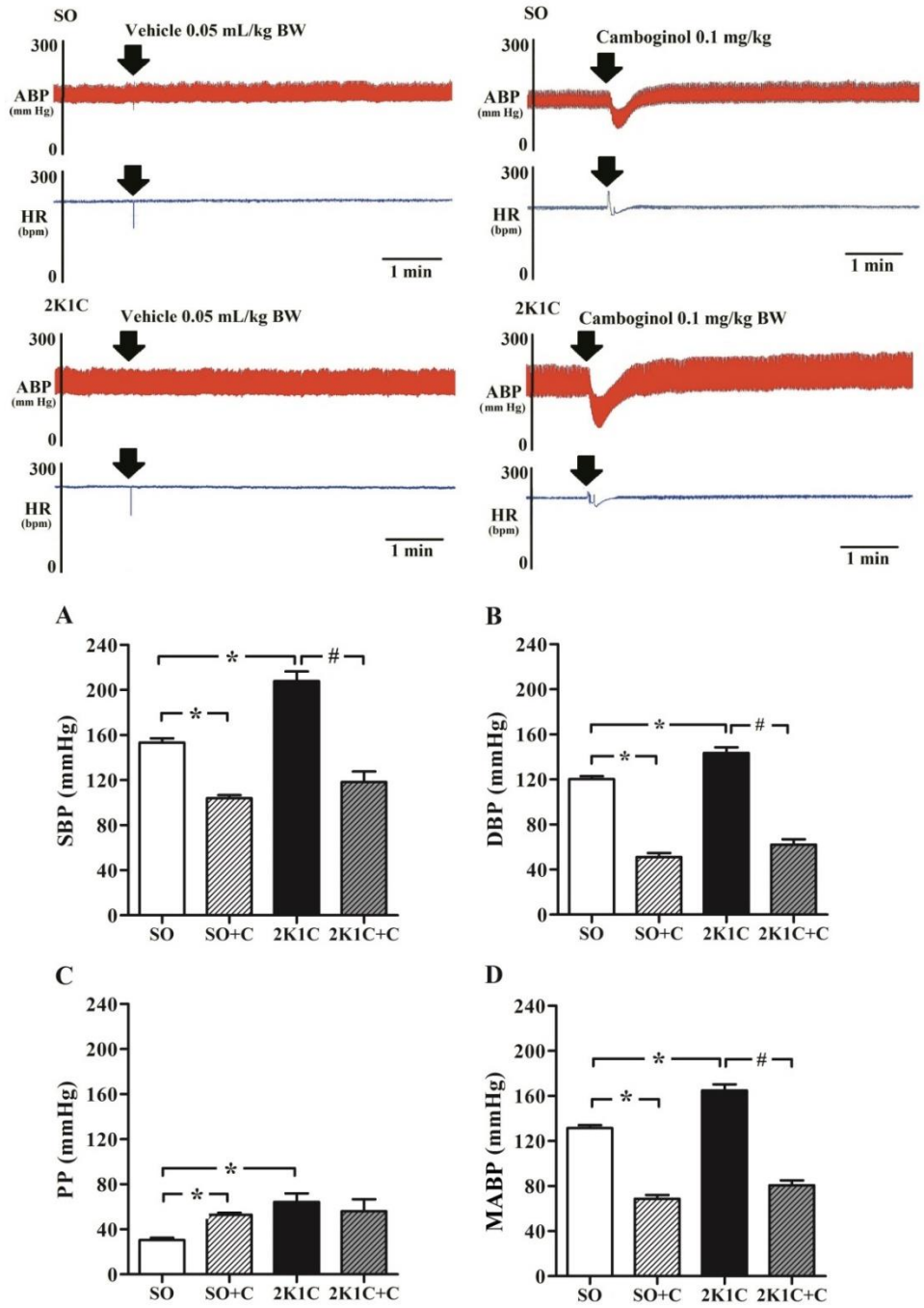

Figure 2. Acute hypotensive effects of camboginol (C) 0.1 mg/kg BW in the 2-kidneys-1-clip (2K1C) and sham operation (SO) groups (n=6 each). Upper panels show the recorded tracing of arterial blood pressure (ABP) and heart rate (HR) and lower panels represent A) systolic blood pressure (SBP), B) diastolic blood pressure (DBP), C) pulse pressure (PP), and D) mean arterial blood pressure (MABP). Data are mean±S.E.M. $^{*,\#}$ P<0.05 compared with SO and 2K1C group, respectively (one-way ANOVA with Newman-Keuls post-hoc test).

The bolus injection of 0.1 mg/kg BW camboginol in the SO rats significantly decreased the SBP, DBP, MABP, and HR compared to the vehicle injection (SBP 104±3 vs. 153±4, DBP 51±4 vs. 120±3, MABP 69±3 vs.131±3 mm Hg, and HR 154±9 vs. 210±5 bpm, respectively; P<0.05). In 2K1C rats, the injection of 0.1 mg/kg BW camboginol also decreased the SBP, DBP, MABP, and HR significantly compared to the vehicle injection (SBP 118±9 vs. 208±9, DBP 62±5 vs. 144±5, MABP 81±4 vs. 165±5 mm Hg, and HR 178±3 vs. 212±5 bpm, respectively; P<0.05). These results suggested the hypotensive potential of camboginol.

3.3 Effect of camboginol on BRS

After intravenous injection of six consecutive doses of PE and SNP, changes in the HR in response to the changed MABP and the calculated BRS of 2K1C were significantly lower than those of the SO rats at all respective experimental doses ($P<0.05$) (Figure 3). This finding suggested an impaired BRS in the 2K1C rats. However, camboginol treatment restored this impairment by increasing the HR and hence the BRS in response to both of the PE and SNP effects.

3.4 Effect of camboginol on plasma MDA level

The levels of plasma MDA in the 2K1C rats were significantly higher than the SO rats (64.3±8.9 vs. 30.0±2.5 µmol/L, $P<0.05$, respectively) (Figure 4). Camboginol treatment significantly lowered the plasma MDA level in the 2K1C rats to 31.3±11.4 µmol/L, $P<0.05$). This finding suggested the *in vivo* free radical scavenging property of camboginol.

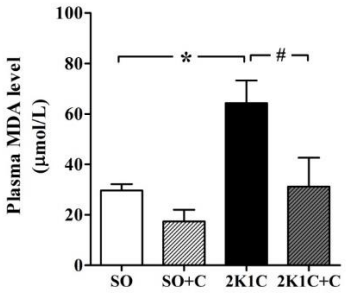

Figure 4. Effect of camboginol (0.1 mg/kg BW + 5 µg/min/kg BW) on plasma malondialdehyde (MDA) levels in 2-kidneys-1-clip (2K1C) hypertensive and sham operative (SO) normotensive rats (n=6 each). Data are mean±S.E.M. $^{*,\#}$ $P<0.05$ compared with SO and 2K1C groups, respectively, (one-way ANOVA with Newman-Keuls post-hoc test).

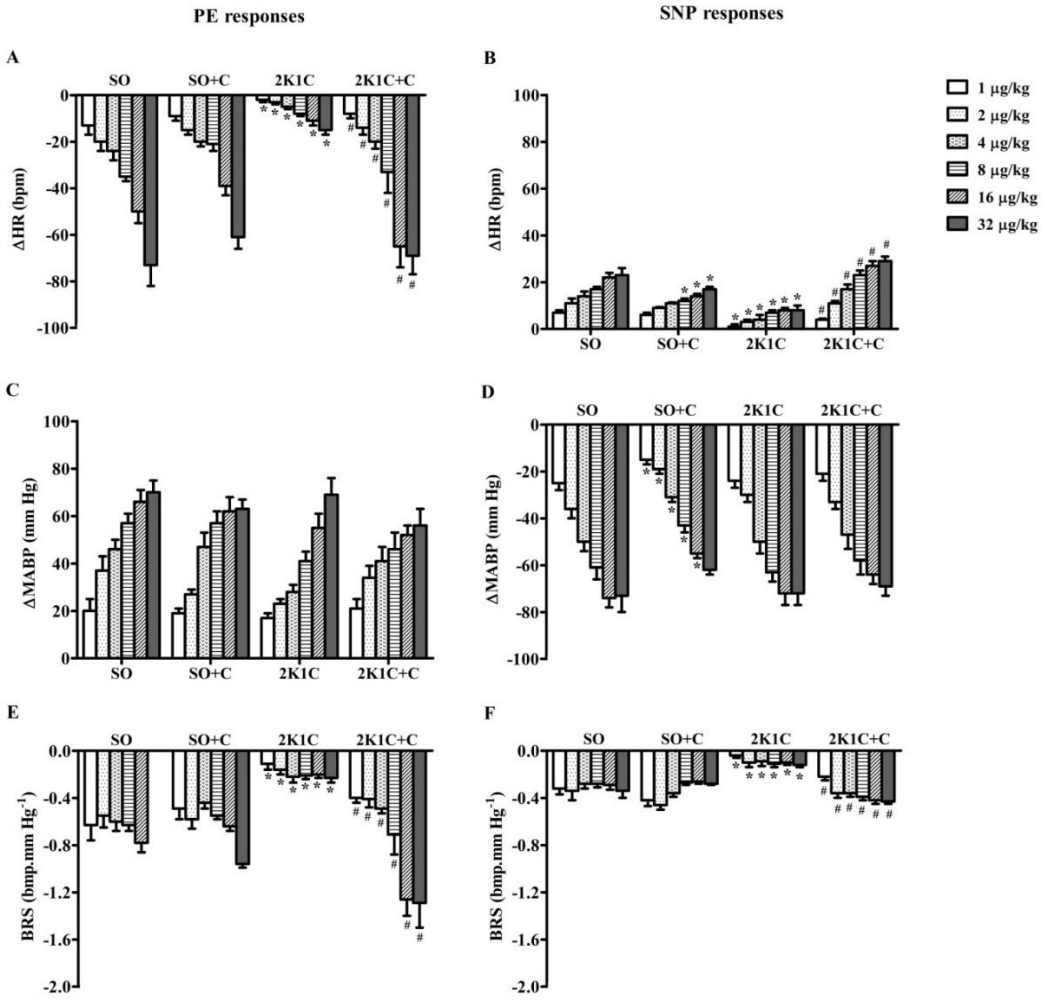

Figure 3. Baroreflex sensitivity (BRS) in response to either phenylephrine (PE left panel) or sodium nitroprusside (SNP right panel) in 2-kidneys-1-clip (2K1C) and sham operation (SO) group (n=6 each) during treatment with camboginol (C=0.1 mg/kg BW + 5 µg/min/kg BW). BRS=ΔHR/ΔMABP. Data are mean±S.E.M. $^{*,\#}P<0.05$ compared with the SO and 2K1C groups at the respective concentrations of PE or SNP (one-way ANOVA with Newman-Keuls post-hoc test).

3.5 Vasorelaxant effect of camboginol in isolated thoracic aortic ring

The addition of the cumulative doses of camboginol (10^{-13}-10^{-5} M) significantly relaxed the PE-precontracted endothelium-intact aortic rings of both 2K1C and SO groups in a concentration-dependent manner (2K1C pD_2 = 8.01±0.66 and SO pD_2 = 9.67±0.19; P<0.05) (Figure 5A). Denudation of the functional endothelium completely abolished camboginol-induced vasorelaxation in both groups (Figure 5B). This suggested that the vasorelaxant effect of camboginol is endothelium-dependent.

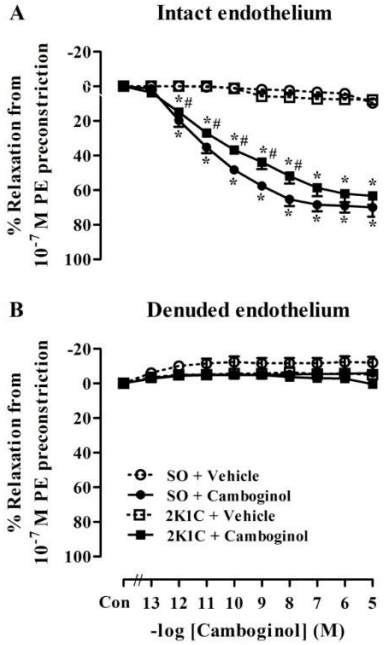

Figure 5. Effects of camboginol (10^{-13}-10^{-5} M) or vehicle (0.1-0.9% DMSO) on vasorelaxation of A) endothelium-intact and B) endothelium-denuded aortic rings from 2-kidneys-1-clip (2K1C) group or sham operative (SO) group (n=6 each). Values are mean±S.E.M of percentage relaxation from 10^{-7} M phenylephrine (PE) pre-contraction. $^{*, \#}$ P<0.05 compared with respective vehicle groups and SO + camboginol group (one-way ANOVA with Newman-Keuls post-hoc test).

3.6 Effect of specific inhibitors on camboginol-induced vasorelaxation

Pretreatment of the endothelium-intact aortic rings with 10^{-4} M L-NAME significantly and completely abolished camboginol-induced vasorelaxation in both SO and 2K1C rats (Figure 6A and B). In the presence of 10^{-6} M indomethacin and 10^{-5} M glibenclamide, the relaxation of the SO aortic rings by addition of camboginol were partial abolished (Figures 6C and E). However, in the presence of 10^{-6} M indomethacin, the percent relaxation of the aortic rings from the 2K1C rats was not abolished (Figure 6D) compared to the SO rats which suggested that the endothelial PGI_2 synthesis might be impaired in the 2K1C rats. In contrast, the percent relaxation of the aortic rings from the 2K1C rats in response to camboginol in the presence of 10^{-5} M glibenclamide was similar to the SO rats (Figures 6E and F) which suggested that partial involvement of vascular smooth muscle K_{ATP} in this signaling vasorelaxation was present in both groups. Pretreatment of the endothelium-intact aortic rings from the SO and 2K1C rats with 10^{-3} M TEA did not affect the percent relaxation response to camboginol (Figures 6G and H). These findings suggested that the vasorelaxant mechanism of camboginol action in the SO thoracic aorta involves mainly NO-signaling pathways with partial involvement of PGI_2 and K_{ATP}. In the 2K1C rats, the contribution of NO-signaling and partial K_{ATP} and K_{Ca}-involved pathways were observed.

3.7 Effect of camboginol on eNOS expression

2K1C rats exhibited a significant reduction of eNOS expression in aortic endothelium compared with the control rats (Figure 7). Treatment with camboginol was able to enhance protein expression of aortic eNOS in the 2K1C rats. This finding further supported the mechanism of vaso-relaxation by camboginol which involves the endothelial NO-signaling pathway.

4. Discussion and Conclusions

2K1C is the most relevant model that has the characteristics of human RVH which involves unilateral stenosis of the renal artery. This leads to a permanent reduction in renal perfusion pressure in one kidney resulting in increased AII production (Goldblatt, Lynch, Hanzal, & Summerville, 1934; Navar *et al.,* 1998). In this study, four weeks after experimental renal artery stenosis, SBP, DBP, PP, and MABP of the 2K1C rats measured under pentobarbital sodium anesthetization were significantly higher than those of the SO rats which suggested that the development of hypertension was successful. These results were also supported by the significant increase in the cardiac mass and the atrophy of the clipped left kidney with the hypertrophy of the non-clipped right kidney of 2K1C compared to the SO rats.

It was found that an acute intravenous administration of camboginol showed a significant transient reduction in SBP, DBP, MABP, and HR in 2K1C and SO rats suggesting the hypotensive potential of this plant compound. This hypotensive effect was not sustained during the infusion of our experimental dose (0.1 mg/kg BW + 5 µg/min/kg BW) in both 2K1C and SO rats. One of the explanations of this may be due to the well regulated baroreflex in the SO and the improved BRS in the 2K1C rats caused by camboginol. The selected dose of camboginol in this *in vivo* study was possibly not high enough to maintain its hypotensive action compared to candesartan which is a specific AII receptor antagonist previously reported (Hiranyachattada *et al.,* 2005).

However, the acute bradycardic effect of camboginol may involve NO action on the sinoatrial node firing rate since NO has been shown to exert a biphasic effect depending on its concentration. NO could increase HR by activating hyperpolarization-activated pacemaker current (I_f) at a low concentration but it could potentially decrease HR by inhibition of the L-type calcium current at a high concentration (Mani, Nahavandi, Moosavi, Safarinejad, & Dehpour, 2002). Moreover, it is likely that camboginol may

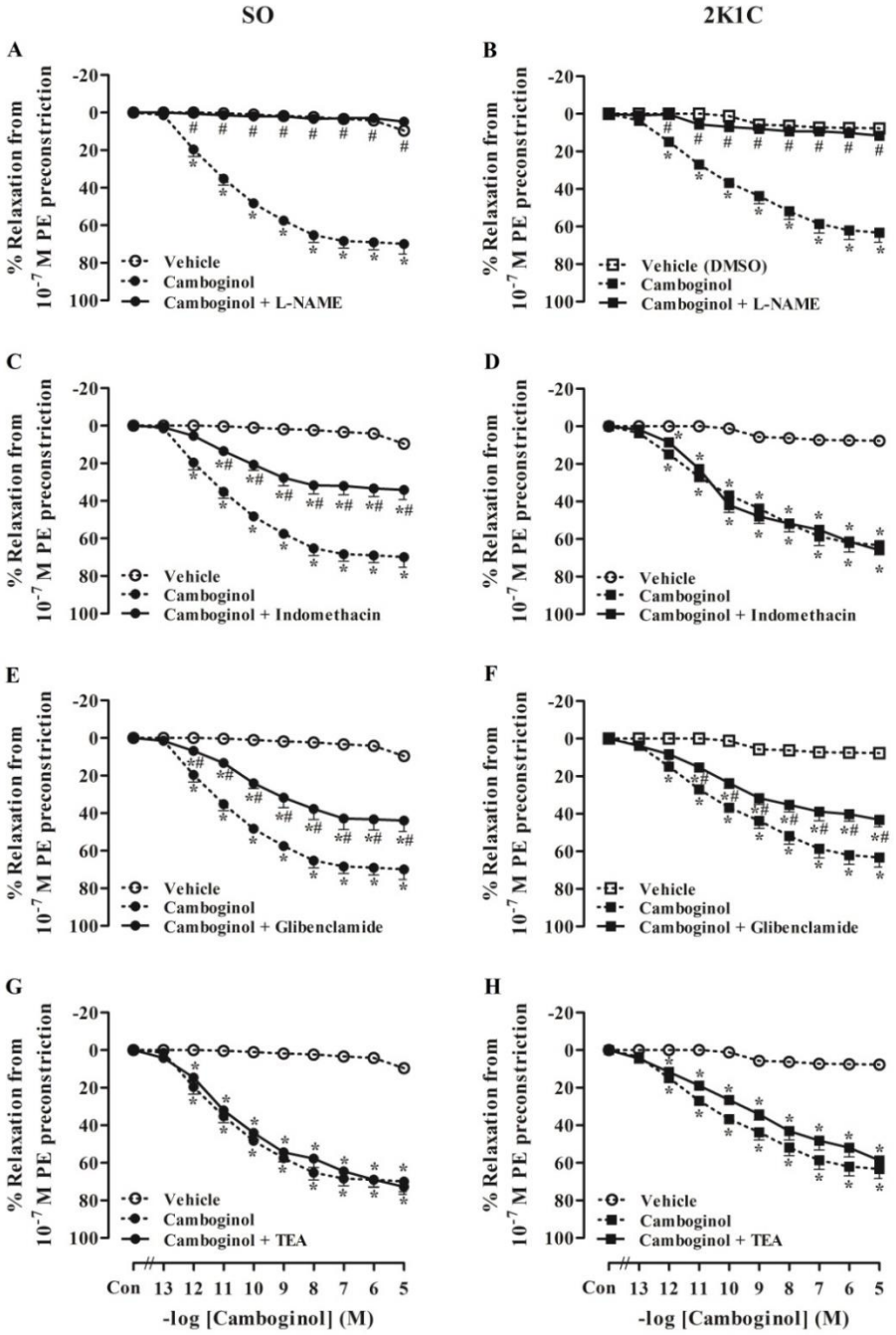

Figure 6. Effects of camboginol (10^{-13}-10^{-5} M) on vasorelaxation of endothelium-intact aortic rings from sham operative (SO left panels) and 2-kidneys-1-clip (2K1C right panels) groups (n=6 each) in the presence of specific inhibitors: A&B) 10^{-4} M L-NAME; C&D) 10^{-6} M indomethacin, E&F) 10^{-5} M glibenclemide, and G&H) 10^{-3} M TEA. Values are mean±S.E.M. $^{*,\#}$ $P<0.05$ compared with vehicle and camboginol groups respectively, (one-way ANOVA with Newman-Keuls post-hoc test).

act directly on the nucleus tractus solitarii (NTS), the cardiovascular regulating center in the medulla oblongata, to cause bradycardia since the overexpression of eNOS was reported in the NTS which consequently reduces sympathetic nerve activity, HR, and ABP in conscious rats (Sakai *et al.,* 2000).

2K1C rats also exhibited the impairment of BRS which was possibly due to the inability to increase the HR in response to a decreased ABP and vice versa. It was proven that an impairment of BRS in RVH was related to an overproduction of O_2^- by NADPH oxidase enzyme (Botelho-Ono *et al.,* 2011; Queiroz, Guimarães, Mendes-Junior, &

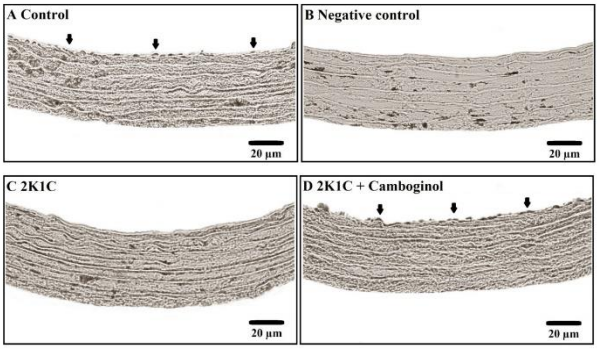

Figure 7. Endothelial nitric oxide synthase (eNOS) expression in aortic endothelium of control and 2-kidneys-1-clip (2K1C) rat; A) control (n=2), B) negative control (n=2), C) 2K1C (n=2) and D) after camboginol (0.1 mg/kg + 5 µg/min/kg BW) in 2K1C (n=3). Brown staining in aortic endothelium represents eNOS in the aortic wall. Black staining represents nucleus of vascular smooth muscle cell. Arrow indicates the eNOS location. 400X, Scale bar=20 µm.

Braga, 2012). Acute administration of Vitamin C or apocynin, a NADPH oxidase inhibitor, prominently improved the blunt BRS in 2K1C rats (Botelho-One *et al.*, 2011). Likewise, it is likely that the antioxidant property of camboginol may improve the impairment of the 2K1C autonomic function.

The rise in the plasma level of MDA confirmed the oxidative stress status in 2K1C rats in this study. Camboginol treatment suppressed the elevated plasma MDA in 2K1C rats which supports the robust antioxidant property of camboginol that was previously reported by Yamaguchi *et al.* (2000a, 2000b) and Hutadilok-Towatana *et al.* (2007).

The percent relaxation of the thoracic aorta from the 2K1C rats by cumulative addition of camboginol showed lower pD_2 compared to the SO rats which suggested that a higher concentration of camboginol was required to induce the vasorelaxation to the similar degree as in the SO rats. The mechanisms of camboginol action in the SO aorta were found to be endothelial dependent involving mainly NO generation and partially both PGI_2 production and activation of the vascular K_{ATP} channel. However, in the 2K1C rats, the camboginol actions occurred mainly through NO generation and partial K_{ATP} channel to a lower extent than in the SO rats (Figure 6). This may be due to an impairment of endothelial function in the 2K1C rats. However, it is unlikely that the vasorelaxant effect of camboginol could occur via vascular smooth muscle K_{ATP} or K_{Ca} stimulation since the denudation of both the SO and 2K1C aortic rings completely abolished the relaxation (Figure 5).

Our immunohistological study indicated the obviously lower eNOS expression in the 2K1C rats compared to the control. This finding was similar to previous reports by Sánchez *et al.* (2006) and Ulker, McMaster, McKeown, and Bayraktutan (2003) that showed the eNOS expression significantly decreased in the dysfunctional arteries from the hypertensive rats. An overproduction of O_2^- reduced bioactive NO by promoting NO inactivation and by promoting eNOS uncoupling (Förstermann, 2010). Camboginol treatment augmented eNOS expression in the vascular endothelium of the 2K1C rats. This finding further supported the mechanism of camboginol action in the 2K1C rats that might occur via eNOS expression in the vascular endothelium.

It is concluded that the vasorelaxant mechanisms of camboginol action in 2K1C hypertensive rats were likely to be endothelial dependent involving NO production and the opening of the vascular K_{ATP} channel similar to normotensive rats but with a lesser degree. The partial involvement of PGI_2 production in the vasorelaxant mechanisms by camboginol in normotensive disappeared in the 2K1C rats. The robust antioxidant activity and the hypotensive potential of camboginol may be beneficial in long term treatment as an anti-hypertensive supplement. However, an application of this plant benzophenone as an alternative treatment of hypertension requires further pharmacological studies regarding its effects in the resistant vessels and intensive cellular mechanisms of its action.

Acknowledgements

This research was financially supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission [SC75705685], the PSU-Ph.D. Scholarships**,** the Graduate School Research Support Funding for Thesis and Faculty of Science Research Fund, Academic Year 2015, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

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