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Vasodilatory Effects of Aqueous Extract from *Harungana madagascariensis* Stem Bark in Isolated Rat Aorta: The Roles of Endothelium and K^+ Channels

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Abstract

Background: Hypertension is the most modifiable risk factor for cardiovascular diseases worlwide. It is one of the leading causes for mortality, as it may be asymptomatic but a lot of complications will develop rapidly and leading to death. Prevention and control of hypertension decreases mortality, and heart failure. The aim of this study was to investigate the vascular activity of aqueous extract obtained from stem bark of *Harungana madagascariensis* (Hypericaceae) and to determine the pharmacological mechanisms of the extract on rat aorta *in vitro*.

Methods and Findings: The activity of different concentrations of *H. madagascariensis* aqueous extract (HMAE) was evaluated on contractile responses of isolated aorta to phenylephrine (PE, 1 µM) and potassium chloride (KCl, 60 mM). Then, various pharmacological agents were used to assess the involved vascular mechanisms. The extract (10^{-3}- 8.10^{-1} mg/mL and 6.10^{-1}-1 mg/mL) induced a concentration dependent relaxation respectively in aortic rings precontracted by PE and KCl. The effect on PE-preconstricted aortic rings was significantly reduced after endothelium removal, whereas it was enhanced on KCl-preconstricted rings. L-NAME, methylene blue and indomethacin as well as tetraethylammonium and barium chloride reduced significantly this vasorelaxation after PE-induced contraction. The vasorelaxant effect of sodium nitroprusside was not modified in the presence of HMAE.

Conclusions: The involvement of NO-cGMP as well as prostacyclin-cAMP pathways contributes to the endothelium-dependent relaxant effects of HMAE after PE-induced contraction. Contribution of Ca^{2+}-activated K^+ channels, voltage-activated K^+ channels and K^+ inward rectifier channels as endothelium-independent mechanisms could also explain its vasorelaxants effects. The observed data suggest that HMAE has potential effects as phytoalternative treatment for hypertension and other cardiovascular diseases.

Keywords: *Harungana madagascariensis*; Vasorelaxant effect; Endothelium-dependent; Potassium channels; Hypertension; Rat.

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Introduction

Non communicable diseases (NCDs) were reported to cause 38 million (68%) of 56 million human deaths worldwide in 2012. The four main NCDs are cardiovascular diseases (CVDs, 46% of all NCDs deaths), cancers (22%), respiratory diseases (10.7%) and diabetes (4%) [1]. Arterial hypertension (AH) is one of the prevalent CVDs [2]. By 2025 the number of hypertensive patients around the world will reach 25% of adults [3]. Uncontrolled AH is considered as a major risk factor for stroke, myocardial infarction, ischemia, blindness and kidney diseases. In Sub-Saharan Africa, AH has been on the rise with reports indicating higher values in urban settings compared to rural settings [4,5]. The development of synthetic drugs for hypertension treatment has increased.

Important classes of antihypertensive drugs are sympatholytic drugs, diuretics, Ca^{2+} channel blockers, angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, and vasodilators [6]. Antihypertensive drugs influence arterial blood pressure at four effector sites: the resistance vessels, the capacitance vessels, the heart, and the kidney [7]. Because these drugs are effective but also cause many side effects, approximately 80% of the world population uses various herbal medicines because of their low toxicity and better acceptability by the human body [8]. Thus, many vasodilator compounds derived from plants such as resveratrol [9], chlorogenic acid [10] as well as alkaloids, terpenoids and flavonoids have been identified [11].

Harungana madagascariensis Lam. ex Poir belong to the family 'Hypericaceae', earlier known as 'Guttiferae' and is commonly known as haronga, orange-milk or dragon's blood tree [12]. It is native to Madagascar, Mauritius and tropical Africa growing on margins of wet forests [13]. Preparations from different parts of *H. madagascariensis* locally called 'atondo' in Ewondo (a tribe of Center Cameroon) have been used for the treatment of a wide spectrum of human diseases. The bark is employed in the treatment of malaria, river blindness, ulcer, asthma, hepatitis, dysmenorrhea, toothache and hypertension. The leaves of *H. madagascariensis* are used for the treatment of chest pains and urogenital infections. Fruits are used as an abortive [14]. Documented studies indicate isolation of bioactive compounds like anthrones, anthraquinones, xanthones, flavonoids, and essential oils from this plant [15,16]. Scientific validation of the reported medicinal uses indicate that *H. madagascariensis* posses antidiarrhoeal and antibacterial effects [17]; antioxidant effects [18]; anti-inflammatory, anti-plasmodial, antidiabetic and analgesic activities [19-21]. Recently, we demonstrated that *H. madagascariensis* exert hypotensive and cardioprotective effects [22,23]. To date, the effect of *H. madagascariensis* stem barks on the changes of the vascular tone has not been studied yet. Therefore, this study was aimed to investigate for the first time, the effects of stem bark aqueous extract of *H. madagascariensis* on isolated rat thoracic aortic rings as well as the possible mechanisms involved.

Materials and Methods

Plant material and extraction

Fresh *H. madagascariensis* stem barks were collected at Essezok, Mbalmayo (Center Region, Cameroon) in June 2016. The identification of the plant was done at the Cameroon National Herbarium, where a voucher sample was deposited under the registration number No. 4224 HNC. Bark pieces were dried under room temperature and powdered with the help of electrical grinder. One kilogram of powder was introduced into 12 L of distilled water and boiled for 20 minutes. The resulting decoction was filtered through Whatman paper No. 3 and further lyophilized. A crude brown extract powder (HM extract, 98.90 g) was obtained, giving a yield of 9.89%.

Animals

Adult male *Wistar* rats weighing 200-250 g were used. The animals were housed in cages at 22 ± 5°C temperature and given tap water and standard diet *ad libitum*, while a 12 h on/12 h off light cycle was maintained. All experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care and with institutional guidelines.

Preparation of aortic rings

Animals were anaesthetized by intraperitoneal injection of ketamine/diazepam (50/10 mg/kg, i.p). Then, the thorax was opened and the thoracic aorta was quickly removed and cleaned from adherent connective tissues and cut into rings (2-3 mm in length). All the dissection procedures were performed with extreme care to protect the endothelium from inadvertent damage. Aortic rings were suspended horizontally between two stainless steel hooks. One of the hooks was fixed to the chamber wall, while the other was attached to an isometric force transducer (it50, EMKA Technologies, France). Isometric contractile responses were determined by placing the rings in an organ bath (20 mL) (Organ Bath, 2 channels, EMKA Technologies, France) containing Krebs–Henseleit solution (37°C, pH 7.4) of the following composition (mM): NaCl 118, KCl 4.65, $CaCl_2$ 2.52, $MgSO_4$ 1.64, KH_2PO_4 1.18, $NaHCO_3$ 24.9 and glucose 12 and gassed with oxygen. Tissues were then maintained under 2 g initial tension and allowed to equilibrate for 1 h during which bath solution was replaced every 15 min [24]. In some aortic rings, the endothelium was removed by gently rubbing the inner surface with a rough steel needle, and used as the denuded endothelium aorta. In all experimental groups, after equilibration, the presence of a functional endothelium was assessed on the basis of more than 70% relaxation evoked by the endothelium–dependent dilator acetylcholine (ACh, 10^{-6} M) in aorta rings precontracted with phenylephrine (10^{-6} M).

Effects of *H. madagascariensis* extract on phenylephrine or potassium chloride-induced aortic contraction

For the study of the vasodilatory effects of *H. madagascariensis*, the thoracic aorta rings isolated from rats were contracted by using phenylephrine (10^{-6} M, PE) or potassium chloride (60 mM, KCl) in two separate experiments. When the vasoconstriction curves for the rings reached the plateau phase of maximum tension, the extract was cummulatively added in the organ bath at the respective concentrations of 10^{-3}, 4×10^{-3}, 8×10^{-3}, 10^{-2}, 4×10^{-2}, 8×10^{-2}, 10^{-1}, 4×10^{-1}, 6×10^{-1} and 8×10^{-1} mg/mL for the contraction induced by PE 6×10^{-1}, 6.5×10^{-1}, 7×10^{-1}, 7.5×10^{-1}, 8×10^{-1} and 1 mg/mL for the contraction induced by KCl. The tensions were recorded and the vasorelaxant effect of *H. madagascariensis* was calculated as a percentage of the relaxation in response to PE and KCl on the aortic rings.

Effects of *H. madagascariensis* on endothelium-intact or endothelium-denuded aortic rings pre-contracted with phenylephrine or potassium chloride

To examine the role of endothelium on the vasorelaxing effect of *H. madagascariensis*, the vasodilatory effect of *H. madagascariensis* on constriction induced by PE or KCl in rat thoracic aortas was

also evaluated for both intact endothelium rings and denuded endothelium rings. To confirm that the endothelial layer had been removed, we selected the rings with a maximum relaxation induced by acetylcholine less than 30% after phenylephrine (10^{-6} M)-induced contraction in denuded vessels.

Studies of endothelium-related vasorelaxing mechanisms of *H. madagascariensis*

To determine the endothelium-underlying mechanisms, the role of nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) was determined using the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and the guanylate cyclase inhibitor, methylene blue (MB) respectively. Endothelium-intact rings were incubated with L-NAME (10^{-4} M), or MB (10^{-5} M) for 30 min before the addition of PE. The extract of *H. madagascariensis* was then added cumulatively and the concentration-response curves were constructed for the inhibitory responses.

Effect of *H. madagascariensis* on endothelium-intact aortic rings pre-incubated with various K^+ channel blockers

In order to know the role of K^+channels on the extract-induced relaxation, intact aortic rings were pre-incubated with tetraethylammonium chloride (TEA, 10^{-5} M), a blocker of big Ca^{2+}-activated K^+ channels and barium chloride ($BaCl_2$, 10^{-5} M), an inward-rectifier K^+ channel inhibitor for 30 min before PE (10^{-6} M)-induce contraction. Then the extract (10^{-3}, 4.10^{-3}, 8.10^{-3}, 10^{-2}, 4.10^{-2}, 8.10^{-2}, 10^{-1}, 4.10^{-1}, 6.10^{-1} and 8.10^{-1} mg/mL) was added and cumulative concentration-response curves were obtained.

Effect of *H. madagascariensis* on endothelium-intact aortic rings pre-incubated with indomethacin

To determine if prostanoids were involved in the relaxant effect of *H. madagascariensis* (10^{-3}-8.10^{-1} mg/mL) endothelium-intact aortic rings were incubated with indomethacin (10^{-5} M), a non-selective cyclooxygenase inhibitor for 30 min prior to precontraction with PE (10^{-6} M). Then the extract (10^{-3}-8.10^{-1} mg/mL) was added and cumulative concentration-response curve was obtained.

Effect of *H. madagascariensis* on sodium nitroprusside induced vasorelaxation

In order to investigate if the extract could affect the response of the vascular smooth muscle to NO through the soluble guanylyl cyclase (sGC) signaling pathway, we evaluated the vasorelaxant response of the blood vessels in the presence of a NO donor sodium nitroprusside (SNP), which activates the sGC pathway alone, and after incubating with the extract (EC_{50}, 8.10^{-2} mg/mL) and PE induced pre-contraction.

Chemicals and drugs

All chemicals were of analytical grade commercially available. Phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), Nw-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), methylene Blue (BM), tetraethylammonium chloride (TEA), Baryum chloride ($BaCl_2$), were obtained from Sigma-Aldrich (Germany). The stock solutions of these chemicals were prepared by dissolving with distilled water, at concentrations of 10^{-2} M. The solutions were prepared fresh on the day of experiments.

Data analysis

All data are expressed as mean ± SEM. Two pharmacological parameters were determined from the concentration–response curves (analyzed by nonlinear regression (curve fit) using GraphPad Prism (Version 5.0, GraphPad Software, San Diego, CA, USA): The EC_{50}, the negative logarithm of EC_{50} (concentration required to achieve a half-maximal response), and E*max* expressed as the percentage (%) of the maximal effect of the test substance. Statistical comparisons were made using two-way ANOVA followed by the Bonferroni's test. *P* values less than 0.05 were considered to be statistically significant.

Results

Effect of *H. madagascariensis* on rat aorta rings precontracted with phenylephrine or potassium chloride

The *H. madagascariensis* extract induced concentration-dependent relaxation in endothelium-intact aortic rings precontracted by PE or KCl. The maximum relaxant effect (E*max*) was 76.3 ± 2.2% (EC_{50}=8.10^{-2} mg/mL) and 45.0 ± 3.3% (EC_{50}=6.7

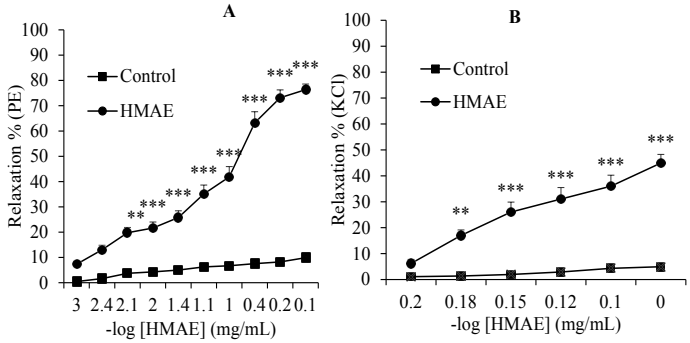

Figure 1 The concentration-dependent relaxation effect of *H. madagascariensis* aqueous extract (HMAE) on PE (1 µM) (A) or KCl (60 mM) (B) on PE (1 µM) (A) or KCl (60 mM) (B) pre-contracted aortic rings. Values are expressed as mean ± SEM (n=6-12). **P<0.01, ***P<0.001 vs. control.

× 10^{-1} mg/mL) at the concentrations of the extract of 8.10^{-1} mg/mL and 1 mg/mL respectively (**Figure 1A and 1B**). In this study, we found the optimal concentration to generate a complete dose-response for *H. madagascariensis* by studying the results of several tests after PE or KCl-induced contraction.

Role of endothelium in *H. madagascariensis*-induced relaxation on aortic rings precontracted with PE or KCl

In this series of experiments, we examined the vasorelaxing effect of *H. madagascariensis* on vascular smooth muscle using endothelium-denuded rat aortic rings. As illustrated in **Figure 2**, *H. madagascariensis* induced relaxation in a concentration-dependent manner for endothelium-intact and denuded vessels. However, removal of the endothelium cause dual effect in blood vessels when contracted with PE or KCl: A significant decrease (P<0.001) in the vasorelaxation produced by *H. madagascariensis* was observed, with an E*max* of 42.4 ± 2.2% in denuded vessels *vs*. 76.3 ± 2.2% in intact rings when contracted with PE (**Figure 2A**). Surprisingly, the removal of endothelium enhanced the relaxation induced by *H. madagascariensis* when rings were pre-contracted with KCl. In this case, the E*max* was 87.0 ± 10.2% in denuded rings *vs*. 45.0 ± 3.3% in intact rings (P<0.001) (**Figure 2B**).

Participation of the NO-cGMP pathway on the vasorelaxant effect of *H. madagascariensis*

Pre-treatment of intact aortic rings with L-NAME (10^{-4} M, a non selective NOS inhibitor) or methylene blue (MB, 10^{-5} M, a guanylate cyclase inhibitor) produced a significant change (P<0.001) of the response and vasorelaxation was markedly inhibited (**Figure 3**). In the presence of L-NAME or MB the E*max* was 3.0 ± 1.2% and 33.3 ± 7.3%, respectively *vs.* 76.3 ± 2.2% in the control aortic rings.

Role of cyclooxygenase on *H. madagascriensis* induced relaxation on aortic rings with endothelium

In the presence of indomethacin (10^{-5} M, a non-selective cyclooxygenase inhibitor) the relaxation induced by the aqueous extract of *H. madagascariensis* was significantly decreased on aortic rings precontracted with PE. The *Emax* decreased from 76.3 ± 2.2% to 45.3 ± 6.0% respectively in absence and in the presence of the indomethacin (**Figure 4**). This represents an

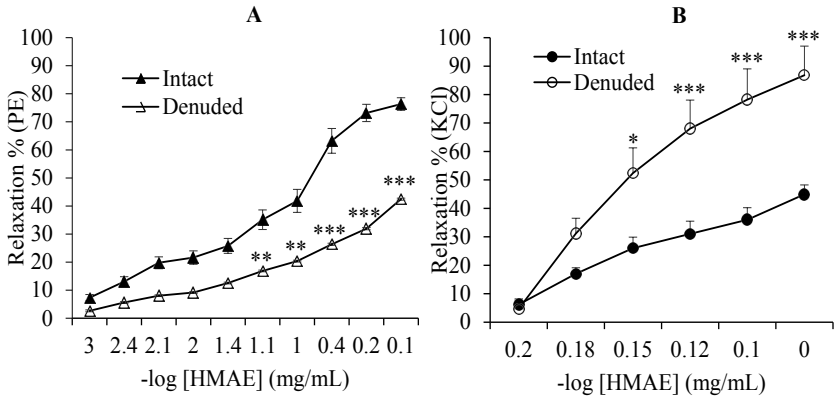

Figure 2 The concentration-dependent relaxation effect of *H. madagascariensis* aqueous extract (HMAE) on PE (10^{-6}M) (A) or KCl (60 mM) (B) pre-contracted aortic rings with (intact) or without (denuded) endothelium. Values are expressed as mean ± SEM (n=8-12). *P<0.05, **P<0.01, ***P<0.001 *vs*. intact aortic rings.

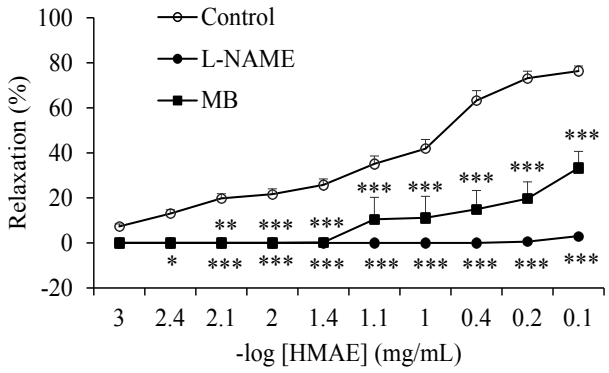

Figure 3 The effects of L-NAME (10^{-4} M) and methylene blue (MB, 10^{-5} M) on cumulative concentration response of *H. madagascariensis* aqueous extract (HMAE, 10^{-3}-8.10^{-1} mg/mL) in intact aortic rings pre-contracted with PE (10^{-6}M). Values are expressed as mean ± SEM (n=6-12). *P<0.05, **P<0.01, ***P<0.001 *vs*. control.

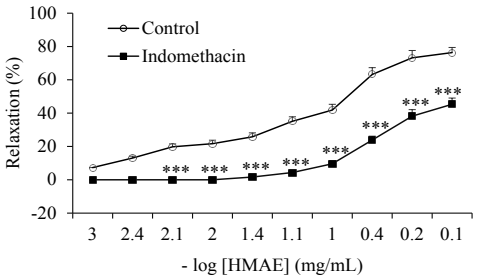

Figure 4 The effect of indomethacin (10^{-5} M) on cumulative concentration response of *H. madagascariensis* aqueous extract (HMAE, 10^{-3}-8.10^{-1} mg/mL) in intact aortic rings pre-contracted with PE (10^{-6} M). Values are expressed as mean ± SEM (n=6-12). ***P<0.001 *vs*. control.

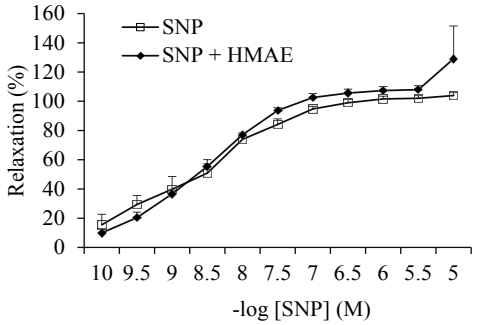

Figure 5 Effect of *H. madagascariensis* aqueous extract (HMAE, 8.10^{-2} mg/mL) on the SNP-induced relaxation of rat aortic rings pre-contracted with PE (10^{-6} M). Values are expressed as mean ± SEM (n=6-11).

inhibition of 31% (P <0.001).

Effect of *H. madagascariensis* on the concentration-relaxant effect of SNP

As shown in **Figure 5**, the relaxation responses of the vessels to SNP were similar in the absence as well as in the presence of the extract.

Role of potassium channels in *H. madagascariensis*-induced relaxation of aortic rings

The vasorelaxant effect of the aqueous extract of *H. madagascariensis* was significantly (P<0.001) inhibited both in the presence of tetraethylammonium (TEA, 10^{-5} M), a non-selective calcium-dependent wide K^+ channel blocker, and in the presence of barium chloride ($BaCl_2$, 10^{-5} M), an inward-rectifier K^+ channel inhibitor. The *Emax* decreased from 76.34 ± 2.2% in the absence of antagonists to 53.61 ± 5.2% and 30.18 ± 2.8% respectively in the presence of TEA and $BaCl_2$ (**Figure 6**).

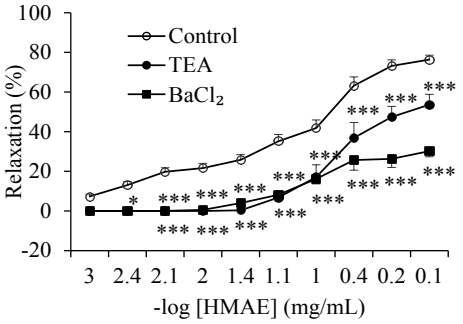

Figure 6 Cumulative concentration-response curves to *H. madagascariensis* aqueous extract (HMAE, 10^{-3}-8.10^{-1} mg/mL) on endothelium-intact aortic rings pre-contracted with PE (1 µM) in the absence (control) or the presence of TEA (10^{-5} M), or barium chloride (10^{-5} M). Values are expressed as mean ± SEM (n=6-12). *P<0.05, ***P<0.001 *vs*. control.

Discussion

The results of the present study showed that *H. madagascariensis* stem bark extract elicits concentration-dependent relaxation on endothelium-intact aortic rings pre-contracted by PE or KCl. This observation demonstrates that *H. madagascariensis* aqueous extract (HMAE) possess a direct vasodilatory effect on vascular smooth muscle. Added to the fact that HMAE has recently been shown to exert hypotensive effect [22] and considering the increasing interest in traditional medicines, *H. madagascariensis* may represent a potential candidate for the treatment of hypertension.

The mechanisms of contraction involved in response of arterial smooth muscle cells to PE and KCl are different. PE is an α_1 adrenoceptor agonist which induced contraction through various calcium entry mechanisms or channels such as receptor-operated calcium channels (ROCCs), capacitative calcium entry (CCE) by the activation of store operated calcium channels (SOCCs), reversal mode of sodium calcium exchangers (NCX), and non-capacitative calcium entry (NCCE) via the activation of diacyl glycerol (DAG) lipase [25,26]. KCL induced contraction mainly by Ca^{2+} influx upon the cell membrane depolarization which activates voltage-dependent L-type Ca^{2+} channels (VDCCs) [27]. The vasorelaxing effect of HMAE was more potent in aortic rings preconstricted with PE than those preconstricted with KCl, suggesting that the blockade of ROCCs may be an important factor in the relaxation induced by HMAE.

The endothelium plays a critical role in determining vasotone, and receptors located on the endothelial surface are primary targets for initiating vasodilatory effects [28,29]. In this study, we observed a dual effect depending on the contracting agent used after endothelium removal in aortic rings. In the presence of KCl, the vasorelaxant effect of HMAE was enhanced in denuded rings compared to intact rings while in denuded rings pre-constricted with PE, the relaxant effect of HMAE was significantly reduced. It seems that in the presence of KCl after endothelium removal, endothelium derived-contracting factors (EDCFs) which counteracts vasodilation in response to vasodilator agents are eliminated, thus resulting in an enhancement of vasodilation due to the absence of EDCFs or, endothelium removal activated some putative factor to enhance relaxation in reponse to HMAE. These

findings are similar with those reported in isolated rat thoracic rings precontracted with norepinephrine, where lidocaine induced-relaxation was significantly enhanced by endothelial removal [30]. And also with those reported in rat mesenteric arteries constricted with methoxamine, where endothelium removal augments the vasodilation of some agents such as isoprenaline, sodium nitroprusside and 8-bromo-cGMP [31].

It is well known that the endothelium releases vasorelaxant agents such as endothelium derived-relaxing factors (EDRF) and prostacyclin (prostaglandin I_2) which induces vasorelaxation of vascular smooth muscle cells and modulates vascular tone [32]. The major EDRF is nitric oxide (NO). It is induced by nitric oxide synthase (NOS) and leads to the enhancement of vasorelaxation through NO-cGMP pathway via guanylate cyclase activation [33]. In endothelial cells, the calcium-calmodulin complex stimulates NO synthase (NOS), which later activates NO formation from L-Arginine. NO then enters the smooth muscle cells and stimulates guanylate cyclase, which increases intracellular cyclic guanosine monophosphate (cGMP). The increase in intracellular cGMP then stimulate cGMP-dependent protein kinases leading to a decrease in the calcium concentrations in the smooth muscle cells, which causes its relaxation [34]. In this study, to better understand the effect of HMAE in denuded ring after PE contraction, we further used NOS and guanylate cyclase inhibitors. The vasorelaxant effect of HMAE was abolished by NOS inhibitor, Nw nitro-L-arginine methyl ester (L-NAME) and significantly attenuated by guanylate cyclase inhibitor, methylene blue. Prostacyclin produced by cyclooxygenase-1 from arachidonic acid increases cAMP, which leads to vascular smooth muscle relaxation [35]. These results show that the cyclooxygenase inhibitor, indomethacin, also inhibited the vasorelaxation induced by HMAE in isolated rat thoracic aorta rings. Therefore, we suggest that the vasorelaxant activity of HMAE may be exerted mainly via the NO-cGMP pathway and partially via the prostacyclin-cAMP pathway.

Sodium nitroprusside (SNP), an endothelium-independent relaxant agent is considered as one of the NO donors most widely studied [36]. Its effect is attributed to its direct action on the vascular smooth muscle (VSM). HMAE did not have any effect on the relaxation elicited by sodium nitroprusside (SNP) after PE-induced contraction, suggesting that endothelium-dependent mechanisms are more likely involved in the vasorelaxant effect of HMAE.

The change in K^+ channel activity is an important mechanism of vasoconstriction and vasodilation as it changes the activity of voltage-dependent Ca^{2+} channels [37]. To investigate the possibility that the vasorelaxant effects of HMAE are mediated via K^+ channels, TEA (a Ca^{2+}-activated K^+ channel blocker and non-specific voltage-activated K^+ channel blocker) and $BaCl_2$ (inwardly rectifying K^+ channel blocker) were used [38,39]. The vasorelaxant effect of HMAE was significantly attenuated by pre-treatment with TEA and $BaCl_2$, suggesting that the relaxant response of HMAE was involved in the role of K^+ channel opening of Ca^{2+}-activated K^+ channels, voltage-activated K^+ channel and K^+ inward rectifier channels in vascular smooth muscle cells.

Conclusions

The aim of this study was to evaluate the vascular effects of HMAE and to characterise the mechanisms in isolated rat aorta. HMAE induced vasorelaxation in rat aortic rings precontracted by either PE or KCl. The involvement of NO-cGMP as well as prostacyclin-cAMP pathways contributes to its endothelium-dependent relaxant effects after PE-induced contraction. Ca^{2+}-activated K^+ channels, voltage-activated K^+ channels and K^+ inward rectifier channels were also involved in the vasorelaxant effect of HMAE. The observed data suggest that HMAE has potential effects on the regulation of the hypertension. More studies are required to determine its anti-hypertensive effects in hypertensive animals and to elucidate the signaling mechanisms. In addition, further studies are needed to characterize the active compound responsible for its cardiovascular properties.

Conflicts of Interest

The authors declare no conflict of interest.

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