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Mechanism of vasorelaxation induced by *Tridax procumbens* extract in rat thoracic aorta

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ABSTRACT

Background/Aim: *Tridax procumbens* (Linn) (Asteraceae) is one of the herbs widely distributed in many parts of the world. Its leaves have long been used for the treatment of hypertension in Nigeria. Previous studies have shown that aqueous leaves of *T. procumbens* extract (TPE) lowers blood pressure through endothelium-dependent and -independent mechanism in the aortic rings isolated from normotensive rats. The aim of the present study was to further investigate mechanisms of TPE-induced relaxation in the aortic artery by assessing its mechanistic interactions with nitric oxide (NO) synthase, cyclic guanosine monophosphate (cGMP), and cyclic adenosine monophosphate (cAMP). **Materials and Methods:** The aortic artery isolated from healthy, young adult normotensive Wistar albino rats (250-300 g) were pre-contracted with phenylephrine (PE) (10^{-7} M) and KCl (60 mM) and were treated with various concentrations of aqueous extract of TPE (0.5-9.0 mg/ml). The changes in arterial tension were recorded using Ugo Basile model 7004 coupled to data capsule acquisition system model 17400. The interaction between TPE with cAMP and cGMP inhibitors was also evaluated. **Results:** The results showed that the TPE (0.5-9.0 mg/ml) significantly ($P < 0.05$) reduced the contraction induced by PE in a concentration-dependent manner. The vasorelaxant effect caused by the TPE was significantly ($P < 0.05$) attenuated with pre-incubation of cGMP (Rp-8Br PET cGMPS) and cAMP (Rp-AMP) inhibitor, respectively. **Conclusion:** These results suggest that TPE causes vasodilatory effects in a concentration-dependent manner in the isolated rat aortic artery. The mechanism of action of TPE is complex. A part of its relaxing effect is mediated directly by blocking or modulating cGMP and cAMP.

KEY WORDS: Cyclic adenosine monophosphate inhibitor, cyclic guanosine monophosphate inhibitor, Rp-8Br PET cyclic guanosine monophosphate, *Tridax procumbens*, vasorelaxant effect

INTRODUCTION

Hypertension is one of the most prevalent and important health problems affecting millions of people in developing as well as developed countries [1]. Although effective synthetic drugs for the treatment of hypertension exist, there remains a great interest in the use of natural plant extracts to attenuate the risk of cardiovascular disorders especially hypertension [2]. Many traditional medicinal herbs such as *Crocus sativus* petals [3], *Berberis vulgaris* root extract [4], *Schisandra chinensis* fruits [5], and many others have been reportedly used for the control of hypertension. However, many of these herbs have not been fully subjected to standard scientific evaluations to verify their potency.

Tridax procumbens L. (Asteraceae) is a common weed that grows in open places, *coarse-textured* soils of tropical regions throughout the world [6]. *T. procumbens* extract (TPE) has been for many years used in Nigeria as an antihypertensive agent [7]. However, the exact mechanisms responsible for its antihypertensive activity are still not fully understood.

Previous studies reported that TPE caused bradycardia and hypotension in normotensive rats [7] and that aqueous leaf of TPE produced relaxation of isolated rat aorta [8]. It was suggested that the relaxation may be partly due to inhibition of Ca^{2+} influx through receptor-gated channels [9]. In the present study, we investigate the role of cGMP and cAMP on the vasodilatory effects of TPE. Furthermore, its effects on the nitric oxide (NO) release were also investigated.

MATERIALS AND METHODS

Ethical Considerations

Experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the Lagos State University College of Medicine and also conformed to the 1985

guidelines for laboratory animal care of the National Institute of Health (NIH).

Plant Material

Fresh leaves of *T. procumbens* were collected from open grassland of Lagos State University College of Medicine, Ikeja, Lagos, Nigeria. Identification of the plant was carried out by a Taxonomist of the Forestry Research Institute, Mr. K.A Adeniji. Following identification, a specimen voucher number FHI 1008876 of the plant was deposited in the herbarium of the Forestry Research Institute, Ibadan, Nigeria.

Preparation of Extract

The leaves were air-dried at room temperature for a week, the dried leaves were pulverized with a laboratory mortar and pestle and 500 g of the powdered leaves were transferred into a conical flask containing 500 ml of distilled water; the mixture was shaken and allowed to stand for 24 h before filtration using a dry Whatman filter paper into a measuring cylinder. The filtrate was then concentrated by evaporation in a water bath (35-40°C) and stored at 4°C until used.

Animals

Healthy, young adult, male Wistar albino rats weighing 250-300 g were used. The animals were kept and maintained under conventional laboratory conditions of temperature, humidity, and light. The animals were allowed free access to standard pellet diet (Live Stock Feeds Nig. Ikeja, Nigeria) and water *ad libitum*.

Vascular Ring Preparation and Pharmacological Studies

The rats were anesthetized with pentobarbital (60 mg/kg, i.p.). The descending thoracic aorta was excised by midline incision, cleaned of fat and connective tissues, with care taken not to stretch the vessel excessively or to disturb the luminal surface of the rings, to ensure the integrity of the endothelium. The aorta was then cut into small rings (3-5 mm in width) and suspended between two wire stirrups in a jacketed organ bath containing 50 ml of normal Krebs physiological solution of the following composition (mM): NaCl 118.2, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11.7. The bathing solution was bubbled continuously with a mixture of 95% oxygen and 5% carbon dioxide at 37°C. The rings were suspended with a resting tension of 0.8 g for at least 60 min then reduced to 0.6 g with constant changing of Krebs solution (every 15 min) to prevent accumulation of metabolites that could otherwise lead to misinterpretation of results [10]. The isometric tension was recorded by the force-displacement transducer (Model 7004; Ugo Basil Varese, Italy) connected to Data capsule Model 17400 for the isometric contractions. The rings were then progressively stretched to an optimal tension of 1 g and allowed to equilibrate for 60 min. Following the equilibration period, the aortic rings were allowed to achieve maximal tension by repeated exposure (each for 5 min) to

isotonic potassium chloride solution (high K^+ 60 mM). The rings that showed vasoconstriction response induced by phenylephrine (PE) (10^{-7} M) were studied. PE was then washed out, and the tension was returned to the baseline levels before the administration of the drugs. The aortic ring was denuded to remove the endothelial layer in some preparations by inserting a pair of forceps into the lumen of each artery and gently rotating it. The viability of each aortic ring was validated by pre-contraction of PE (10^{-7} M) and relaxed by ACh (10^{-5} M) just before the experiment commenced. Relaxation of $\geq 70\%$ indicated the presence of a functional or intact endothelial layer while the lack of relaxation indicated the successful removal of the layer.

Effects of TPE on Endothelium - Intact and - Denuded Ring Pre-contracted with PE

The endothelium-intact and -denuded arteries were pre-contracted with PE (10^{-7} M). After the tonic responses or contractions became stable, increasing concentrations of TPE (0.3-1.8 mg/ml) were added cumulatively.

To characterize TPE-induced vasodilatation pharmacologically, specimens were pre-treated with various inhibitors including a selective cGMP inhibitor (Rp-8-Br-PET-cGMPS) and the selective cAMP inhibitor (KT-5720).

Measurement of Nitrite Production in Isolated Aorta

Nitrite/nitrate, which is the index of vascular NO production was measured according to the method of Raghavan and Dikshit [11]. Describe briefly, aortic rings (6mm in length) were washed in Krebs solution and divided into different experimental groups, which were treated with control or TPE (0.3-1.8 mg/ml) in the endothelium-intact and -denuded rings incubated at 37°C in a shaker for 30 min in a total volume of 1 ml. After ring, supernatants were obtained by centrifugation, equal volumes of Griess reagent (1% sufanilamide and 0.1% naphthylethylene diamine in 5% phosphoric acid) were added, and the mixture was incubated at 37°C for 30 min. Absorbance was measured at 545 nm to determine total nitrite content. The NO production was calculated by subtracting nitrite concentration seen in the presence of the extract from the control, and this is regarded as NO production from experimental rings. Measurement for nitrite production was expressed as μmole/mg dry weight of tissue.

Drugs

L-PE chloride, potassium chloride (KCL), sodium chloride (9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9, 12-epoxy-1H-diindolo [1,2,3-fg:3', 2',1'-kl]pyrrolo [3, 4-i][1,6] benzodiazocine-10-carboxylic acid, hexyl ester (KT-5720), and 2-Bromo-3,4-dihydro-3-[3,5-O-[(R)-mercaptophos phinylidene]-β-D-ribofuranosyl]-6-phenyl-9H-Imidazo[1,2-a] purin-9-one sodium salt (Rp-8-Br-PET-cGMPS). All were purchased from Tocris, United Kingdom.

Statistical Analysis

Data were analyzed using GraphPad Prism version 5.0 statistical software, and the results were expressed as means ± standard error, where n equals the number of animals from which blood vessels were isolated. The data were analyzed using one-way ANOVA. Dunnett's multiple comparison test was used to identify differences between individual means. The confidence interval was set at 95% so that in all cases, results with a value of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Effects of Graded Doses of TPE on PE and KCl-Induced Contraction

Figure 1 shows the typical tracing of relaxation responses to TPE (0.3-1.8 mg/ml) recorded in aortic ring pre-contracted by PE [Figure 1a] or by KCl [Figure 1b]. The tension developed was significantly reduced by cumulative application of TPE.

Role of Endothelium in TPE-Induced Relaxation

TPE (0.3-1.8 mg/ml) showed a concentration-dependent relaxation effect in both endothelium-intact and endothelium-denuded aortic rings after pre-contraction by PE. However, the functional removal of endothelium did not significantly modify TPE-induced relaxation in PE - pre-contracted aortic rings [Figure 2].

Effect of TPE on the Cyclic Guanosine Monophosphate (cGMP) and Cyclic Adenosine Monophosphate (cAMP) Activities

To assess the role of cGMP and cAMP in the relaxation produced by TPE in aortic rings pre-contracted by PE, we used aortic rings pre-incubated with Rp-8-Br-PET-cGMPS ($30\,\mu$M) or a selective PKG inhibitor KT-5720 ($50\,\mu$M) the selective PKA inhibitor in both endothelium-intact and endothelium-denuded rings as pharmacological tools. Under these conditions, the maximum relaxation produced by TPE in endothelium-intact rings was 46.2 ± 4.5%. Pre-incubation for 30 min with the PKG inhibitor Rp-8-Br-PET-cGMPS produced a significant decrease ($P < 0.01$) from the maximal relaxation elicited by TPE to 12.8 ± 2.7 and 16.7 ± 4.8 % in endothelium-intact and endothelium-denuded, respectively [Figure 3a and b]. Furthermore, pre-incubation with the PKG inhibitor KT-5720 significantly ($P < 0.01$) inhibited the relaxation induced by TPE in both endothelium-intact and -denuded [Figure 4a and b].

Effect of TPE on NO Production

As shown in Figure 5, the production of nitrite in the incubation media of aortic rings were significantly ($P < 0.01$) increased by TPE treatment in a concentration-dependent manner (0.3-1.8 mg/ml), while functional removal of endothelium significantly reduced this effect [Figure 5].
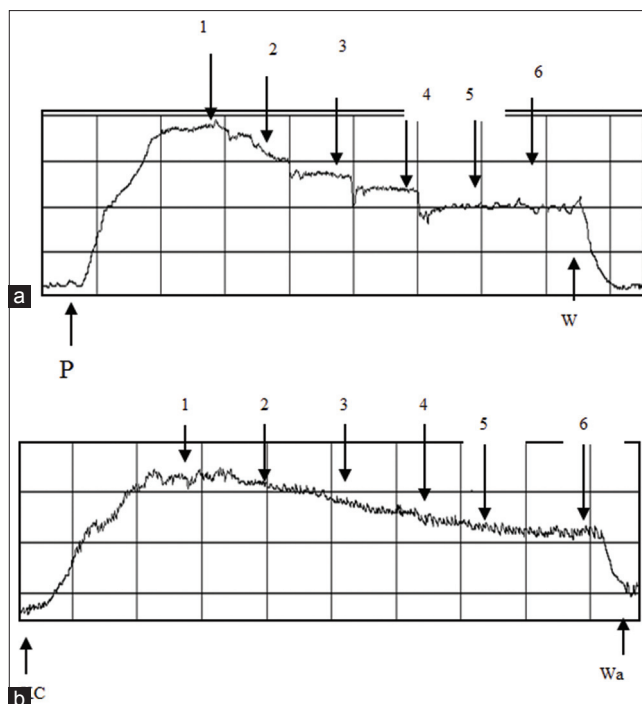

Figure 1: Typical tracing showing the vasorelaxant effects of graded concentration of TPE a (a) Phenylephrine (10^{-7} M) (PE)-induced and (b) (60 mM) KCl-induced contraction in the endothelium-intact aortic ring isolated from normotensive rat. Arrows 1-6 represent cumulatively administered TPE (0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 mg/ml, respectively) administration of drup upward-arrow (P) and washed out at (W) downward-arrow
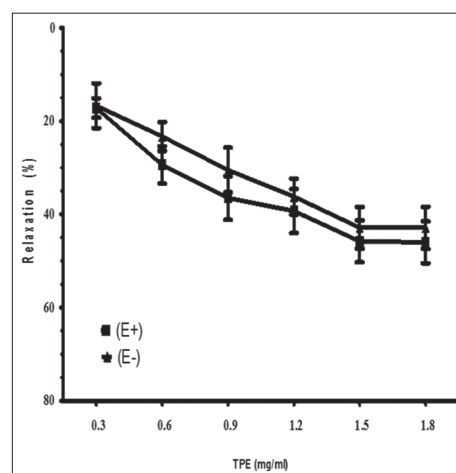

Figure 2: Concentration-response curve showing the vasorelaxant effect of TPE in endothelium-intact (E+) and -denuded (E–) rat aortic rings pre-contracted with phenylephrine (10^{-7} M). (E_{max} = maximal contraction considered as 100%). Each point represent the mean ± standard error (indicated by vertical line) of 6 experiments. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test

DISCUSSION

The data presented in this study provides strong evidence that cyclic GMP/cAMP play a major role in vasorelaxation induced

by TPE in rat aortic smooth muscle. This conclusion was based on the findings that the Rp-8-Br-PET-cGMPS (cGMP inhibitor) and KT-5720 (a cAMP inhibitor) significantly inhibit relaxation induced by TPE at all concentration tested.

Vasodilatation can be facilitated by inhibition of vasoconstriction and secretion of relaxant factors from vascular endothelium. The later is mediated by prostacyclin, bradykinin, and NO. Our previous observation in rats aortic smooth muscle showed that relaxation to TPE was markedly inhibited by blockers of NO and prostacyclin [12]. This would support the idea that cGMP-dependent pathways are involved in mediating relaxation to TPE in this tissue. Indeed, in the present study, TPE dose-dependently increases nitrite concentration in the rat aorta confirming that TPE relaxes the rat aorta via endothelium-dependent NO/cGMP pathways.

The mechanism by which cyclic GMP mediates the TPE-induced relaxation is not fully understood. However, it was suggested that cGMP may affect sequestration of intracellular Ca^{2+} by affecting Ca^{2+} extrusion pumps and hereby decrease the sensitivity for Ca^{2+} [13]. This mechanism may occur without a change in the membrane potential. Thus, the NO-cGMP role in the relaxation effect of TPE needs to be further investigated.

In this study, when the functional endothelium was removed the vasorelaxant action of TPE still persisted in denuded aortic strips.
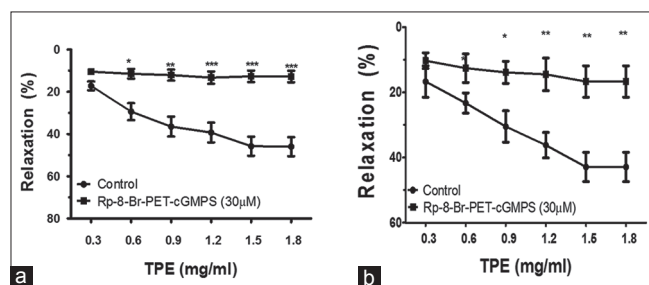

Figure 3: Line graph showing the effects of Rp-8-Br-PET-cGMPS (30 μM) on cumulative concentration response of *Tridax procumbens* extract (0.3 - 1.8 mg/ml) in (a) endothelium-intact (b) endothelium-denuded mesenteric artery pre-contracted with phenylephrine (10^{-7} M). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (*n* = 6) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

This observation indicates that TPE may modulate vascular tone by acting directly on smooth muscle cells via another pathway. It is well known that contractile apparatus of vascular smooth muscle cells depends on the phosphorylation state of the myosin regulatory light chain (MLC) of myosin II, which is phosphorylated by Ca^{2+}/calmodulin-dependent MLC kinase. Phosphorylated MLC interacts with actin filaments, leading to contraction. Conversely, when MLC are dephosphorylated by MLC phosphatase, the interaction between actin-myosin ceases causing relaxation of the muscle [14]. This finding is consistent with results obtained from the previous study that vasorelaxant activity of TPE is mediated through calcium-dependent mechanisms [9].

The results of this study also showed that TPE-induced relaxation of the aortic artery was attenuated in the presence of cAMP inhibitor, and the TPE concentration-response curve was shifted significantly to the right. This indicates that cAMP is involved in the vascular relaxation induced by TPE. The mechanisms by which TPE relaxed the vascular smooth muscle through activation of cAMP signaling pathways are not known. However, reports from several studies indicated that increase in intracellular adenosine 3′, 5′-cyclic monophosphate (cAMP) levels ([cAMP]i), and the subsequent activation of cAMP-dependent signaling pathways leading to the relaxation of vascular smooth muscle can be activated through the following mechanisms; reduced entry of Ca^{2+} from extracellular space through ionic channels [15,16] reduced release of Ca^{2+} from intracellular stores [17]; and decrease of cytoplasmic Ca^{2+} concentration [18-20].

T. procumbens leaves have been reported to contain several active compounds such as alkaloids, flavonoids, quercitin, arachidic, and linoleic acid [21-23]. Earlier studies have reported the presence of dexamethasone, luteolin, and glucoluteolin [24,25], and recently, it was reported that TPE was very rich in linolenic acid [26].

Quercetin is a phytochemical belonging to the flavonoid family and is the most ubiquitous of the dietary flavonoids [27]. Reports indicated that quercetin decreases blood pressure (BP) and/or reduces the severity of hypertension in spontaneously hypertensive rats [28,29]. The flavonoid luteolin also
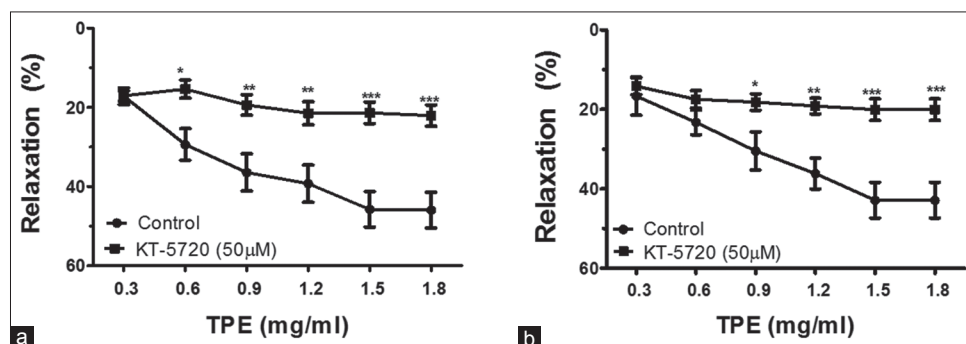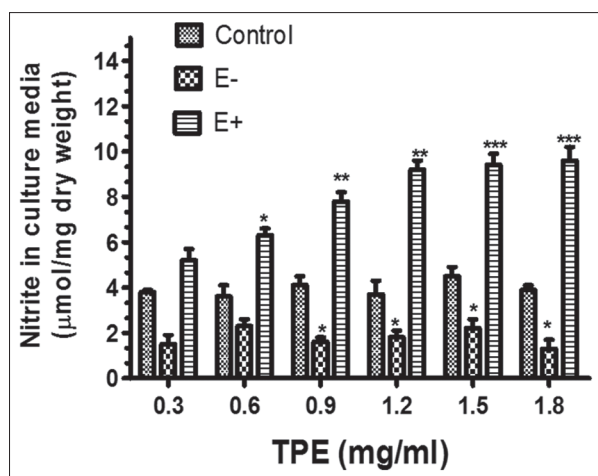

Figure 4: Line graph showing the effects of KT-5720 (50 μM) on cumulative concentration response of *Tridax procumbens* extract (0.3-1.8 mg/ml) in (a) endothelium-intact (b) endothelium-denuded mesenteric artery pre-contracted with phenylephrine (10^{-7} M). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. (*n* = 6) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Figure 5: *Tridax procumbens* extract-induced nitrite production in (E+) endothelium-intact and (E–) endothelium-denuded of rat aorta expressed as µmole/mg dry weight. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. ($n = 6$) (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

induces NO production and arterial relaxation [30]. Thus, vasorelaxant activities of TPE might also result from these active compounds found in them. It has been established by studies that active compounds from the medicinal plant can act as vasodilators [31,32]. Plants showing propensity for NO production are promising candidates for vasodilatation and may have the potential for the prevention and treatment of cardiovascular diseases such as hypertension and atherosclerosis. Further work is necessary to isolate, identify, and characterize more active compound of *T. procumbens* and elucidates the molecular mechanisms of those acting on endothelial and smooth muscle cells.

CONCLUSION

This study investigated the vasorelaxant effect of TPE on isolated rat aorta and its possible mechanisms. The mechanism of its effect involves mainly activation of the NO-cGMP-cAMP pathways and partly by its direct action on the vascular smooth muscle via dephosphorylation of MLC, resulting in vasodilation. This study provides a mechanistic clue to the role of TPE, which has long been used for the treatment of high BP.

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