Effects of Barakol on Vascular Functions in Rats

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Abstract: To investigate the involvement of the endothelium in barakol-induced vasorelaxation, the effect of barakol on vasoactive agents and on extracellular calcium influx in the rat aorta were examined. Moreover, the effect of barakol on endothelial Nitric Oxide Synthase (eNOS) expression in rat aorta was also studied. The effect of cumulative doses of barakol (0.001-1 μM) was examined on the isolated demuced- or intact-endothelium ring preparations. Rings were pretreated with barakol for 30 min before measuring concentration-response curves of vasodilators, carbachol or sodium nitroprusside and vasoconstrictors, methoxamine, 5-hydroxytryptamine and CaCl₂. It was found that barakol caused concentration-dependent vasorelaxation on rat vessels. Pre-treatment with barakol significantly enhanced the vasorelaxation to carbachol (p<0.05). However, barakol did not affect the responses to sodium nitroprusside. Endothelial denudation significantly (p<0.05) inhibited vasorelaxation to barakol at 300 μM. Barakol significantly (p<0.001) reduced the contractions induced by 5-hydroxytryptamine or CaCl₂, but not methoxamine. To investigate the effect of barakol on eNOS expression in rat aorta, rings were incubated with treated or non-treated barakol or DMSO control for 4 and 8 h, respectively, then collected and stored at -80°C until RNA isolation was performed. This result showed that chronic exposure of barakol significantly (p<0.05) inhibited eNOS mRNA expression in dose-dependent manner suggesting that barakol reduced vasorelaxation partially via the endothelium-dependent manner and consequently probably continuous consumption of barakol might not be good for health. Moreover, vasorelaxant effects of barakol involved inhibition of extracellular calcium influx, possibly via receptor-operated calcium channels, also needed further investigation.

Key words: Barakol, rat aorta, vasorelaxation, eNOS, chronic exposure

INTRODUCTION

Barakol is a biologically active compound extracted from leaves and flowers of Cassia siamea. Barakol was originally extracted by Hassanali-Walji et al. (1969). Its chemical structure was identified as 3,4-dihydro-3,8-dihydroxy-2,5-dimethyl-1,4-dioxapherane (C₁₇H₁₀O₄) and its molecular weight is 232 (Bycroft et al., 1970) (Fig. 1). Cassia siamea, a plant has been traditionally used for the treatment of fever, skin disease, constipation, diabetes, asthma, hypertension, diuresis and insomnia (Arttasit, 1987; Bunyaratavanakong, 1992; Kinghorn and Balandrin, 1992; Manosroi, 1994) and it appears to function as an anxiolytic agent in exploratory behavioral activities (Arunlakshana, 1949; Thongsaard et al., 1996). Moreover, it also uses as sedative drug. It has antidepressant-like effect in stressed rat (Wongwitdeecha et al., 2005). In animal studies, barakol has been shown to possess hypotensive activity (Suwan et al., 1992) and serotonergic receptor antagonist activity (Tongroach et al., 1992).

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Barakol has significant effect on cardiovascular function by decreasing systolic and diastolic blood pressure in both endothelial and denuded endothelial rat aorta via atropine inhibition. The mechanism by which barakol decreases blood pressure depends on the animal species (Suwan et al., 1992). Therefore, we are interested in investigating the effect of barakol on vascular activity in rat aorta using various kinds of vasoconstrictors such as methoxamine (MTX), 5-hydroxytryptamine (5-HT) and CaCl₂ and vasodilators such as carbachol (CCh) and sodium nitroprusside (SNP). Moreover, the effect of barakol on eNOS mRNA expression in rat aorta was also studied.

MATERIALS AND METHODS

Barakol extract and purification: In late 2007, barakol was extracted from young leaves of Cassia siamea. The leaves were cut into small pieces and boiled in 0.5% sulfuric acid at 60°C for 2 h. The mixture was filtered, alkalinized with sodium hydrogen carbonate and extracted with dichloromethane. The organic layer was concentrated by evaporating under reduced pressure and shaking with 5% acetic acid and neutralized with 25% ammonium hydroxide. The greenish-yellow solid of crude barakol extract was obtained. Recrystallization from aqueous methanol gave barakol (300 mg). The purity of the compound was confirmed by thin layer chromatography on silica gels and nuclear magnetic resonance (Thongsara et al., 2001).

Effect of barakol on vasoactive agents: In 2007, this whole research project has been approved by the Local Animal Research Ethics Committee before starting. Wistar rats, 250-300 g, were purchased from National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakhonpathom, Thailand. Animals were kept in the Animal Care Center, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand. Animals were fed food and drinking water ad libitum. Light/dark interval and temperature were controlled at 12/12 h cycle and 25°C, respectively.

Male Wistar rats were anaesthetized with Zoletil® (Virbac Laboratories, France, 50 mg kg⁻¹, i.m.). Following thoracotomy, the thoracic aorta was dissected from the rat. The aorta was cleaned of fat and connective tissue and cut into 4-5 mm ring segments. Each ring was transferred to a jacketed organ bath filled with 50 mL of Krebs-Henseleit solution (NaCl 118 mM, KCl 4.7 mM, MgSO₄ 1.2 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 25 mM, CaCl₂ 2 mM, D-glucose 10 mM). The solution in the bath was maintained at a temperature of 37°C and bubbled with 95% O₂ and 5% CO₂ mixture. The solution in the organ bath was exchanged every 15 min for 1 h. The rings were stretched to an optimal passive tension of about 1 g.

Experimental protocol: Following a 1 h equilibration period, methoxamine was used to increase smooth muscle tone. In vehicle-control experiments, dimethyl sulfoxide (DMSO) alone was added cumulatively in the same volumes as those used in the experiments with barakol. In order to investigate the role of the endothelium in responses to barakol, the endothelium was removed by rubbing the luminal surface with a cocktail stick. The preparation was considered to be endothelium-denuded if vasorelaxation to 10 μM carbachol was less than 10% of induced tone.

The effects of barakol on vasoactive agents were investigated in the rat aorta. Aortic rings were pretreated with barakol (10 and 100 μM) for 30 min before concentration-response curves of vasodilators, carbachol (CCh, 0.01-100 μM) or sodium nitroprusside (SNP, 0.0001-10 μM) and vasoconstrictors, methoxamine (MXT, 0.1-300 μM) and 5-hydroxytryptamine (5-HT, 0.1-300 μM) were constructed. In addition, to investigate the calcium inhibitory effect of barakol, concentration-response curves of CaCl₂ (10 μM-30 mM) depolarized by 100 mM KCl were obtained in the absence or presence of barakol at a concentration of 10 and 100 μM.

Effect of barakol on eNOS expression in rat aorta: Forty-eight male Wistar rats, 300-350 g, were fed food and drinking water ad libitum. Light/dark interval and temperature were controlled at 12/12 h cycle and 25°C, respectively. They were anesthetized with Zoletil (50 mg kg⁻¹, i.m.) injected at the right gastrocnemius muscle. To investigate the concentration dependent and time dependent effect of barakol on vasorelaxation in rat thoracic aorta, each experiment group was treated with three different barakol concentrations (10, 30 and 100 μM) and DMSO control, incubated at two different periods of time, 4 and 8 h.

After rats were decapitated, the thoracic aorta was dissected gently, cleaned and immersed immediately in Krebs-Henseleit solution, KHS (in mM; NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 2, D-glucose 10). Each aorta was cut into 4-5 pieces, 4-5 mm ring segments and immersed in sterilized petri dish containing KHS treated or non-treated with barakol according to the experimental design. Stock barakol solutions were prepared in DMSO. Then the dissected rat aortic rings in the prepared mixture were incubated in a 37°C incubator with 95% O₂ and 5% CO₂ for 4 and 8 h, respectively.
Determination of RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR): Thereafter incubated, rat aortic rings were collected in sterilized eppendorfs and stored at -80°C. RNA isolation was performed using PureZOL RNA Isolation Reagent (Biorad, USA), oligonucleotide primers (Sigma Aldrich Company) and reverse transcriptase (synthesized from Vivantis). Total RNA was isolated from the rat aorta according to the recommendations of the manufacturer (Biorad, USA). Rat β-actin was used as an internal control (GenBank: NM_031144). β-actin upper primer is 5’ CCAAGACGAGAAAAGCCT 3’, β-actin lower primer is 5’ GTCTCCGGAGTGGCATCACA 3’. eNOS upper primer is 5’ AGCTGCGATGGAACACTTGA 3’, eNOS lower primer is 5’ CAGCACATCCAAAGCGGCCATT 3’ (GenBank: NM_021833). The eNOS mRNA was determined by using 2 steps RT-PCR. For the reverse transcription, 1 µg RNA was converted to cDNA by AMV reversed transcriptase. The reverse transcription reaction was done at 42°C for 1 h. The optimal PCR amplification of β-actin (300 bp) was run for 35 cycles as follows: denaturation at 94°C for 45 sec, annealing at 63°C for 45 sec and extension at 72°C for 60 sec. Moreover, the optimal PCR amplification of eNOS was run for 30 cycles as in the following: denaturation at 94°C for 45 sec, annealing at 63°C for 45 sec and extension at 72°C for 60 sec. Then PCR products were examined by electrophoresis in 1% agarose gel stained with 0.3 µg mL⁻¹ ethidium bromide and photographed with Fotodyne Incorporated, Hartland, WI, USA. The eNOS target band is 740 bp and β-actin band is 300 bp. The eNOS mRNA/β-actin ratio data were derived from absolute eNOS band density divided by β-actin band density by Scion Image software, NIH (http://www.scioncorp.com).

Statistical analysis: Data were expressed as Mean±SEM. The experimental and control groups were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni/LSD post hoc test (SPSS version 11.5) for multiple comparisons, with the level of significance set at 5% (p<0.05).

RESULTS AND DISCUSSION

The effects of barakol on responses to vasodilators: Carbachol (CCh, 0.01-100 µM) induced concentration dependent vasorelaxations in the rat aorta. Pre-treatment of aortic rings with barakol 10 or 100 µM had great tendency but not significant enhancement effects on vasorelaxation to carbachol (control: pD₂ = 6.36±0.10, n = 6; 10 µM barakol: pD₂ = 6.40±0.09, n = 6; 100 µM barakol: pD₂ = 6.66±0.13, n = 6, Fig. 2a). Pre-treatment of aortic rings with barakol (10 and 100 µM) had no effects on vasorelaxation to sodium nitroprusside (SNP, 0.0001-10 µM, control: pD₂ = 7.73±0.04, n = 6; 10 µM barakol: pD₂ = 7.60±0.07, n = 6; 100 µM barakol: pD₂ = 7.59±0.06, n = 6, Fig. 2b).
The effects of barakol on vasoconstrictors and CaCl₂:

Although pre-treatment of aortic rings with barakol (10, 100 µM) did not affect on methoxamine (MTX, 0.1-300 µM) induced vasoconstriction, but it had a great tendency to inhibit the vasoconstriction of MTX (control: pD₂ = 4.71±0.05, n = 6; 10 µM barakol: pD₂ = 4.63±0.11, n = 6; 100 µM barakol: pD₂ = 4.13 ±0.50, n = 6, Fig. 3a). Pre-treatment of aortic rings with barakol (10, 100 µM) significantly inhibited the maximum vasoconstrictions to 5-hydroxytryptamine (5-HT; control: pD₂ = 4.39±0.13, n = 6; 10 µM barakol: pD₂ = 4.42±0.18, n = 6, p<0.001; 100 µM barakol: pD₂ = 5.11±0.40, n = 6, p<0.001, Fig. 3b). Pre-incubation with barakol (10, 100 µM) significantly reduced the maximal contractions to CaCl₂ (10 µM-30 mM) depolarized with 100 mM KCl (control: pD₂ = 3.85±0.08, n = 7; 10 µM barakol = 3.86±0.07, p<0.001, n = 7; 100 µM barakol = 3.77±0.08, p<0.001, n = 7, Fig. 3c).

The study of the mediators involving in barakol effect on 5-HT-induced vasoconstriction: The further studies were to find out any mediators involving the inhibitory effect of barakol on 5-HT-induced vasoconstriction of aortic rings with or without endothelium. In the presence of 100 µM barakol, there was an obvious enhancement of 5-HT-induced contraction of the denuded aortic rings compared with endothelium intact rings (p<0.001), while there was no difference from control DMSO, Fig. 4. When
there was a nitric oxide synthase inhibitor, L-NAME, or cyclooxygenase inhibitor, indomethacin alone, the inhibitory effect of barakol on 5-HT-induced vasoconstriction were not observed compared to those in the absence of an inhibitor. However, in the combination of L-NAME and indomethacin, the effect of barakol on 5-HT-induced vasoconstriction was significantly different from the effect of barakol with L-NAME (p<0.05), or indomethacin alone (p<0.001).

**Effect of barakol on eNOS expression in rat aorta:** The levels of eNOS mRNA (740 bp) of the rat aortic rings incubated with 10, 30 and 100 μM barakol for 4 and 8 h were less than that in controls. From this study, the eNOS mRNA/β-actin ratio was significantly decreased in 100 μM barakol-treated group at 4 h incubation p<0.05 (Fig. 5a) and 8 h incubation p<0.05 (Fig. 5b) compared with control groups. However, there were no alterations of the eNOS expression at 10 and 30 μM barakol at 4 and 8 h incubation compared with control groups.

The present study reports that vasorelaxation of aortic rings to barakol is partly mediated via the endothelium-dependent pathway by possibly releasing Endothelium-Derived Relaxing Factor (EDRF), Nitric Oxide (NO) and prostacycline (PGI). However, chronic exposure to barakol likely inhibited eNOS mRNA expression; thereby decreased NO production from the endothelial
cells leading to impair vasorelaxation. In addition, barakol decreased vasoconstriction caused by CaCl₂-induced depolarization probably by interfering Ca²⁺ influx.

Barakol mediated vasorelaxation partially through endothelium dependence. Earlier study showed that barakol had antihypertensive activity (Ahn et al., 1978). Although, barakol was extensively investigated the effect on central nervous system, it has not been widely known about the effect and mechanism of barakol on vascular function. The present study determined the possible effect and mechanism of action of barakol on rat aorta reactivity. Present results demonstrated that barakol had a trend to cause the vasorelaxation in rat aorta by enhancing the relaxant effect of carbacol (CCh). Moreover, barakol reduced dose-dependently the agonist-induced vasoconstrictions, methoxamine (MTX) and serotonin (5-HT) via α-adrenoceptors and serotonin receptors, respectively. The endothelium contributed to barakol-mediated vasorelaxation because barakol-reduced vasoconstriction induced by 5-HT was absent after endothelial denudation. Furthermore barakol did not alter the direct relaxation responses to the NO donor, sodium nitroprusside, SNP.

The further findings of barakol involved vasorelaxation was the release of EDRF(s). In the presence of barakol, the combination of Nitric Oxide Synthase (NOS) inhibitor, L-NAME and cyclo-oxygenase (COX) inhibitor, indomethacin, enhanced the 5-HT-evoked vasoconstriction compared to L-NAME or indomethacin alone. The results indicated that barakol mediated vasorelaxations in the rat aorta partly by releasing NO and PG1 from the endothelial cells.

The NO, an endothelium-derived relaxing factor, is synthesized from L-arginine by NOS (Moncada et al., 1991a, b; Schini and Vanhoutte, 1991). Two of the major isoforms of NOS have been identified and play important roles on vascular and inflammatory actions. First the constitutive Ca²⁺-dependent NOS isoform is present mainly in endothelial cells (eNOS) and brain. Second the inducible Ca²⁺-independent NOS isoform (iNOS) activated by cytokines is found predominantly in macrophages and smooth muscle cells (Fürstermann et al., 1991). The NO biosynthesis and release appear to be regulated at the transcriptional level and at the level of eNOS action. Various substances such as growth factor and glucose stimulate the expression of eNOS mRNA in endothelial cells (Cosentino et al., 1997; Hood et al., 1998). Interestingly in the present study, the inhibitory effect of barakol on NO production was demonstrated indirectly by reducing eNOS mRNA expression from rat aorta in dose- but not time-dependent manners when aortic rings were pre-incubated with 10, 30, 100 μM barakol at 4 and 8 h. This finding implied that long-term exposure of barakol possibly affected the NO production, upstream of eNOS translation leading to reduce endothelium-dependent relaxation. However, in acute phase, barakol appeared to induce the constitutive NO released from the endothelial cells to contribute to the vascular relaxation tone. In the study of the effect of barakol on the extracellular calcium influx in the rat aorta, we also found that vasorelaxant effects of barakol involved inhibition of extracellular calcium influx, possibly via receptor-operated calcium channels.

In summary, this study showed that vasorelaxation of aortic rings to barakol was partially mediated via the endothelium-dependent pathway by possibly releasing Endothelium-Derived Relaxing Factor (EDRF), Nitric Oxide (NO) and prostacycline (PG1). Long-term exposure to barakol inhibited eNOS mRNA expression; thereby decreased NO production implied that the target site of action of barakol might be upstream of eNOS translation which resulting in the impairment of endothelium-dependent relaxation. However, in short period exposure, barakol appeared to induce the constitutive NO released from the endothelial cells to reduce the vascular tone. In addition, barakol also attenuated vasoconstriction caused by CaCl₂-induced depolarization possibly by interfering Ca²⁺ influx which needed further investigation.

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