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Curcumin and Tetrahydrocurcumin Restore the Impairment of Endothelium-dependent Vasorelaxation Induced by Homocysteine Thiolactone in Rat Aortic Rings

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Abstract: The aim of the present study was to investigate the effects of curcumin and Tetra Hydro Curcumin (THC) on the inhibition of endothelium-dependent vasorelaxation of the isolated rat aorta by Homocysteine Thiolactone (HTL). Carbachol, an endothelium-dependent vasodilator, caused concentration-dependent vasorelaxation in rat aortic rings. Exposure of aortic rings to HTL (0.3 and 1 mM) for 90 min significantly inhibited endothelium-dependent vasorelaxation to carbachol. In addition, contractions induced by methoxamine were significantly reduced after pretreatment with 3 mM HTL. Curcumin (10 and 30 μM) significantly restored carbachol-induced vasorelaxation inhibited by HTL (1 mM). Similar effects were observed after pretreatment of aortic rings with THC (10 and 30 μM). Moreover, HTL-induced impairment of vasorelaxation to carbachol could be blocked by either L-arginine (3 mM), a precursor of nitric oxide or superoxide dismutase (SOD, 200 U mL^{-1}), a scavenger of superoxide anion. These results demonstrate that impairment of endothelium-dependent vasorelaxation induced by HTL is due to a reduction of nitric oxide and the generation of oxygen free radicals. Interestingly, curcumin and THC could restore endothelial dysfunction induced by HTL which may be related to their antioxidant properties. The present study provides pharmacological data to support the hypothesis that curcumin and THC have vasoprotective effects in hyperhomocysteinemia.

Key words: Curcumin, tetrahydrocurcumin, homocysteine thiolactone, vasorelaxation, rat aorta

INTRODUCTION

Homocysteine, a sulfur-containing amino acid, is an intermediate product in metabolism of L-methionine. Deficiencies of vitamin B_{12} and folate cause an increase in plasma level of homocysteine, termed as hyperhomocysteinemia (Alshatwi, 2007). Hyperhomocysteinemia is a powerful independent risk factor for various cardiovascular disease such as atherosclerosis, hypertension, myocardial infarction (Balakumar *et al*., 2007; Joshaghani *et al*., 2007; Laghari *et al*., 2009; Ravari *et al*., 2009; Damorou *et al*., 2010; Williams and Schalinske, 2010). In fact, homocysteine acts as a pro-thrombotic, pro-inflammatory and vasorelaxation-imparing factor (Perla-Kajan *et al*., 2007). Several studies have shown that endothelium-dependent vasorelaxations are impaired in animals and human with hyperhomocysteinemia (Ungvari *et al*., 1999; Boger *et al*., 2000; Tawakol *et al*., 1997; Abahji *et al*., 2007).

There is evidence that Homocysteine Thiolactone (HTL), a homocysteine-reactive product, is involved in vascular damage due to homocysteine (Jakubowski, 2008; Karolczak and Olas, 2009). Protein N-homocysteinylation induced by HTL may lead to cardiovascular disorders (Karolczak and Olas, 2009). Incubation of rat aortic rings with HTL causes an impairment of endothelium-dependent vasorelaxation (Liu *et al*., 2007). The endothelial dysfunctions induced by HTL involve a decreased release of nitric oxide from endothelial cells and increased generation of reactive oxygen species (Liu *et al*., 2007; Jakubowski, 2008; Karolczak and Olas, 2009).

Curcumin (diferuloylmethane) is a phenolic compound from the plant *Curcuma longa* or turmeric. Commonly, it is used as a spices and coloring agent (Srivastava *et al*., 2011). Pharmacological studies have demonstrated that curcumin has a variety of effects, including antispasmodic (Itthipanichpong *et al*., 2003), antidepressant (Yu *et al*., 2002), anti-oxidant (Manikandan *et al*., 2004; Hussein and Abu-Zinadah, 2010; Sivabalan and Anuradha, 2010), antibacterial (Negi *et al*., 1999; Pandey *et al*., 2011),

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anti-inflammatory (Kohli *et al*., 2005; Yuan *et al*., 2006), anticarcinogenic (Yoysungnoen *et al*., 2008), antinociceptive (Tajik *et al*., 2007) Schistosomicidal properties (EL-Sherbiny *et al*., 2006). Moreover, curcumin could lower plasma level of glucose (Sivabalan and Anuradha, 2010). In addition, curcumin could restore endothelial dysfunction (Ramaswami *et al*., 2004). Recent studies in diabetic rats have shown that tetrahydrocurcumin, an active metabolite of curcumin, has anti-oxidant (Murugan and Pari, 2006a), anti-diabetic (Murugan and Pari, 2006b) and anti-hyperlipidemic effects (Pari and Murugan, 2007). However, curcumin and THC have not been studied in endothelial dysfunction induced by HTL. Thus, the aim of this study was to investigate the effects of curcumin and THC against endothelial dysfunction induced by HTL and mechanisms involved in their actions in the isolated rat aorta.

MATERIALS AND METHODS

Chemicals: All drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) but zoletil was purchased from Virbac (Carros Cedex, France). Curcumin and THC were prepared by our laboratory. All drugs were dissolved in the Krebs solution, except curcumin and THC were dissolved in dimethyl sulphoxide.

Extraction of curcumin and tetrahydrocurcumin: The mixture of curcuminoid extracted from the rhizomes of *Curcuma longa* was subjected to silica gel column chromatography, using hexane-dichloromethane, dichloromethane and dichloromethane-methanol as eluents to yield curcumin, the major compound. THC was synthesized from curcumin as described by Yoysungnoen *et al*. (2008). Structures of curcumin and THC were shown in Fig. 1.

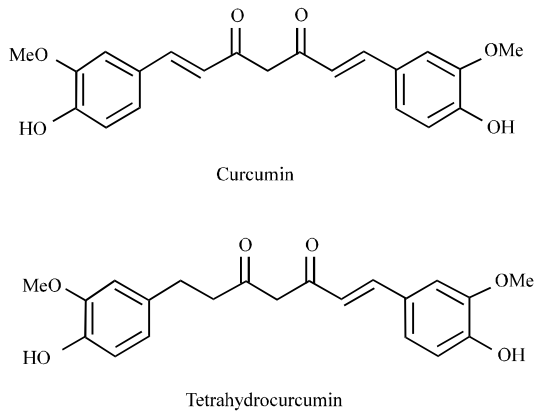

Fig. 1: Structures of curcumin and tetrahydocurcumin

Tissue preparation: In 2010, experiments were performed using aorta obtained from male Wistar rats (300-350 g) bred and kept by the National Laboratory Animal Center, Mahidol University, Thailand. The rats were fed with standard laboratory rat chow and tap water and housed in standard environmental condition (25°C) under 12 h light/dark cycles. All experiments were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

The rats were anaesthetized with zoletil 50 mg kg^{-1} (tiletamine chloridrate and zolazepan chloridrate). Into quadriceps muscle and killed by cervical dislocation (Tep-areenan and Sawasdee, 2011). Following a 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 mounted between two stainless wires and then transferred to a jacketed organ bath filled with 20 ml of modified Krebs-Henseleit solution (composition, mM; NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10) that was maintained at 37°C and bubbled continuously with 95% O_2 and 5% CO_2 mixture. The buffer in the organ bath was exchanged every 15 min for 1 h. The rings were mounted between two triangular stainless steel hooks that were passed through the lumen and stretched to an optimal passive tension of about 1 g and then allowed to equilibrate for 60 min before experiments were started. Tension was measured by isometric force transducers (MLT 0210) connected to a MacLab recording system (AD instruments, New South Wales, Australia).

Experimental protocol: Following a 1 h equilibration period, aortic rings of control were incubated with vehicle (distilled water) and the rings of HTL groups were incubated with HTL (0.3 and 1 mM) for 90 min. After 90 min of incubation, methoxamine, an alpha adrenoceptor agonist, was used to increase vascular tone by approximately 1 g. Once a stable tone was achieved, concentration-response curves of carbachol (1 nM-100 μM) were constructed.

To investigate the effects of HTL on contractions induced by methoxamine, after aortic rings were allowed to equilibrate for 1 h at 1 g tension, aortic rings were incubated with vehicle or HTL (0.3 to 30 mM) for 90 min. After incubation period of 90 min, methoxamine (0.1-300 μM) was added cumulatively in the bath.

To investigate the effects of antioxidants, curcumin and THC, on impairment of endothelium-dependent relaxation induced by HTL, curcumin (10 and 30 μM) and THC (10 and 30 μM), were co-incubation with 1 mM HTL for 90 min. In addition, the effects of L-arginine, a

precursor of nitric oxide (NO) and SOD, a scavenger of superoxide anion, on inhibition of HTL were investigated. L-arginine (3 mM) and SOD (200 U mL^{-1}) were co-incubation with 1 mM HTL for 90 min. This concentration of HTL was used as methoxamine could not induce tone after incubation of aortic rings with a higher concentration (3 mM) of HTL. After incubation of 90 min, tone was induced by addition of methoxamine. Then, concentration-response curves of carbachol were constructed.

Statistical analysis: The concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) and maximal responses (R_{max}) were obtained from the concentration-response curve fitted to a sigmoidal logistic equation using the GraphPad Prism package as described by Tep-areenan *et al.* (2003). R_{max} and pEC_{50} values (negative logarithm of the EC_{50}) were compared by analysis of variance (ANOVA) with statistically significant differences between groups being determined by Bonferroni's post-hoc test. Results are expressed as mean+SE. A value of $p<0.05$ was considered statistical significant. The number of animals in each group is represented by n (Tep-areenan and Sawasdee, 2011).

RESULTS

Effects of HTL on relaxation and contraction of rat aortic rings: In Fig. 2, contractions induced by 30 μM methoxamine were significantly ($p<0.001$) inhibited in rings incubated with 3 mM HTL (R_{max}: control = 1.04±0.15 g, n = 6; 3 mM HTL = 1.04±0.15 g, n = 6) but not 0.3 and 1 mM HTL. In addition, 10 aortic mM or 30 mM of HTL completely inhibited methoxamine-induced contraction (data not shown).

Carbachol caused concentration-dependent relaxation (R_{max} = 101±3% with EC_{50} = 6.24±0.08, n = 6). Endothelium-dependent vasorelaxations to carbachol were significantly ($p<0.001$) reduced after incubation of aortic rings with HTL (0.3 and 1 mM) (R_{max}: control = 101±3%, n = 6; 0.3 mM HTL = 77.1±3.4%, n = 6; 1 mM HTL = 52.6±3.2%, n = 6, Fig. 3).

Effects of curcumin and THC on endothelium-dependent vasorelaxation to carbachol in rat aortic rings: Treatment of aortic rings with curcumin in different concentrations (10 and 30 μM) significantly ($p<0.001$) prevented the

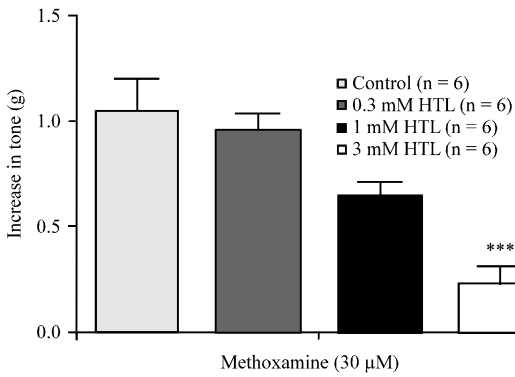

Fig. 2: Effects of pre-treatment with homocysteine thiolactone (0.3 to 3 mM) for 90 min on contractions to methoxamine (30 μM) in rat aortic rings. Data are shown as Mean±SEM
***Significantly different at $p<0.001$

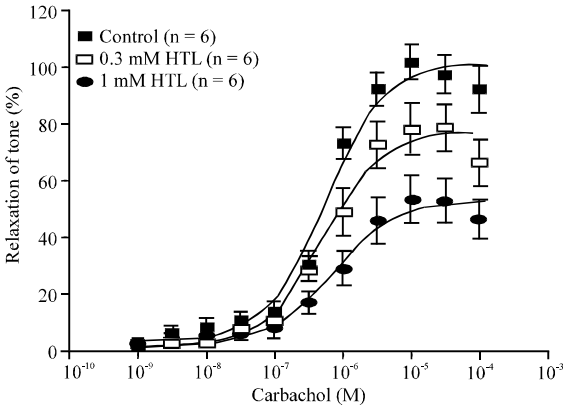

Fig. 3: Effects of pre-treatment with homocysteine thiolactone (0.3 and 1 mM HTL) for 90 min on carbachol-induced vasorelaxation in rat aortic rings. Data are shown as Mean±SEM

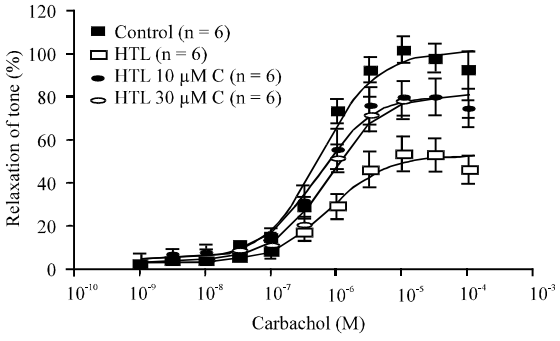

Fig. 4: Effects of pre-treatment with homocysteine thiolactone (HTL 1 mM) for 90 min on carbachol-induced vasorelaxation in the presence of 10 and 30 μM curcumin (C) in rat aortic rings. Data are shown as Mean±SEM

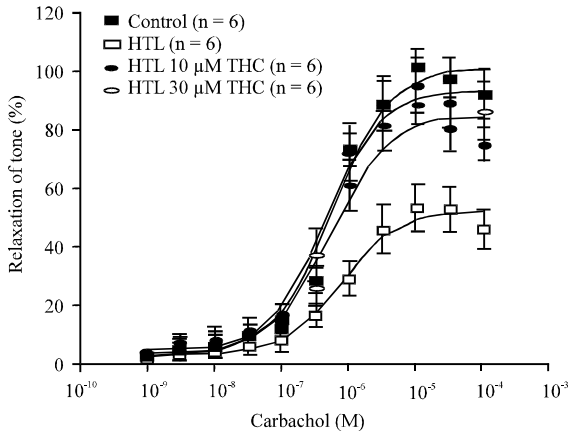

Fig. 5: Effects of pre-treatment with homocysteine thiolactone (HTL 1 mM) for 90 min on carbachol-induced vasorelaxation in the presence of 10 and 30 μM tetrahydrocurcumin (THC) in rat aortic rings. Data are shown as Mean±SEM

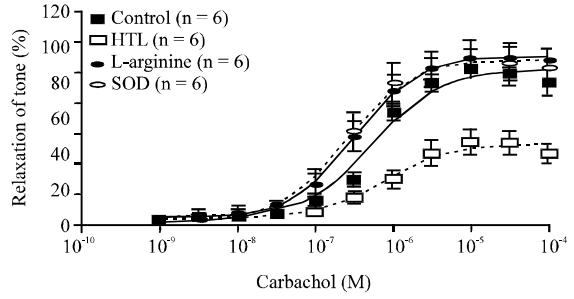

Fig. 6: Effects of pre-treatment with homocysteine thiolactone (HTL 1 mM) for 90 min on carbachol-induced vasorelaxation in the presence of L-arginine (3 mM) and SOD (200 U mL^{-1}) in rat aortic rings. Data are shown as Mean±SEM

inhibitory effects of HTL (1 mM) on relaxant responses to carbachol (R_{max}: control = 101±3%, n = 6; 1 mM HTL = 52.6±3.2%, n = 6; 10 μM curcumin = 80.7±3.4%, n = 6; 30 μM curcumin = 80.9±3.5%, n = 6; Fig. 4). Similarly, THC (10 and 30 μM) significantly ($p<0.001$) restored impairment of relaxation to carbachol after treatment with HTL (1 mM) (R_{max}: control = 101±3%, n = 6; 1 mM HTL = 52.6±3.2%, n = 6; 10 μM THC = 85.1±3.5%, n = 6; 30 μM THC = 93.5±3.9%, n = 6; Fig. 5). However, there was no significant difference of the protective effects between curcumin and THC.

Effects of L-arginine and SOD on endothelium-dependent vasorelaxation to carbachol in rat aortic rings: As shown in Fig. 6, co-incubation of L-arginine (3 mM) or SOD (200 U mL^{-1}) in the presence of HTL (1 mM) significantly restored carbachol-induced vasorelaxation (R_{max}: control = 101±3%, n=6; 1 mM HTL = 52.6±3.2%, n = 6; L-arginine = 109±3%, n = 6; SOD = 108±4%, n = 6).

DISCUSSION

The present study in rat aortic rings demonstrated that HTL inhibited endothelium-dependent vasorelaxation to carbachol and contraction to methoxamine, an alpha adrenoceptor agonist. Interestingly, this is the first time that curcumin and THC have been shown to restore HTL-induced impairment of endothelium-dependent vasorelaxation.

Hyperhomocysteinemia is though to induce arteriosclerosis and peripheral vascular disease which cause dysfunctions of endothelial cells (Abahji *et al*., 2007; Jakubowski, 2008). In the present study, we showed that exposure of aortic rings to HTL (1 mM) caused a significant attenuation of endothelium-dependent vasorelaxation to carbachol. Theses findings are consistent with other studies in isolated animal vessels (Fu *et al*., 2003; Ramaswami *et al*., 2004; Liu *et al*., 2007). In addition, a high concentration of HTL (3 mM) reduced contraction to methoxamine, alpha 1-adrenoceptor agonist. These results suggest that HTL may affect the mechamisms involved in methoxamine-induced contraction, including activation of protein kinase C to increase extracellular Ca^{2+} influx through receptor-operated Ca^{2+} channels and/or Ca^{2+} release from intracellular store (Burt *et al*., 1996; Lyles *et al*., 1998).

Impairment of endothelial functions induced by homocysteine and HTL, a homocysteine-reactive product, involves an increase in the formation of oxygen free radicals, especially superoxide anion and lipid peroxidation products (Zappacosta *et al*., 2001; Fu *et al*., 2003; Ramaswami *et al*., 2004; Jakubowski, 2008). In agreement with previous reports, the present study showed that SOD, a scavenger of superoxide anion, inhibited impairment of endothelium-dependent relaxation induced by HTL in rat aortic rings.

Superoxide amions are known to inhibit endothelium-dependent relaxation by inactivating endothelium-dependent relaxing factors, mainly NO (Mercie *et al*., 2000). Indeed, our results showed that impairment of relaxation induced by HTL are restored after pretreatment with L-arginine, a precursor of NO. These results suggest that endothelial dysfunctions caused by HTL are likely to increase NO degradation by oxygen free radicals and/or decreasing endothelium-derived NO synthesis.

We then investigate the effects of curcumin and its active metabolite, THC, on endothelial dysfunctions induced by HTL. We found that curcumin reverse impairment of endothelium-dependent relaxation induced by HTL in rat aortic rings. These results are in agreement with a previous study showing that curcumin could restore endothelial dysfunctions induced by homocysteine in porcine coronary arteries (Ramaswami *et al*., 2004). Interestingly, we found that THC had similar effects. From the present findings, it is suggested that vasoprotective effects of both curcumin and THC may involve their antioxidant property (Manikandan *et al*., 2004; Murugan and Pari, 2006a; Hussein and Abu-Zinadah, 2010; Sivabalan and Anuradha, 2010). These findings are supported by a recent study showing that curcumin reduced production of superoxide anion in porcine coronary arteries. Moreover, curcumin increase endothelial nitric oxide synthase in porcine coronary arteries (Ramaswami *et al*., 2004). Taken together, mechanisms of the inhibitory effects of curcumin and THC on HTL-induced endothelial dysfunctions may involve decreasing of superoxide anion and increasing production of NO. These may constitute significant mechanisms of cardioprotection by curcumin and its metabolite, THC.

CONCLUSION

These findings demonstrate that curcumin and THC effectively reverse endothelial dysfunction induced by HTL which may be related to scavenging oxygen free radicals and enhancing NO production. The present findings provide pharmacological evidence for mechanisms contributing to vasoprotective effects of curcumin and THC in hyperhomocysteinemia. However, further investigation would need to be pursued to examine other mechanisms including the interaction between THC and HTL.

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