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Crocetin improves endothelium-dependent relaxation of thoracic aorta in hypercholesterolemic rabbit by increasing eNOS activity

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ABSTRACT

Our previous studies have proven that crocetin (CCT), extracted from *Gardenia jasminoides* Ellis, possesses the anti-atherosclerotic effect. Because endothelial dysfunction strongly contributes to the initiation and progression of atherosclerosis, the present study aims to investigate whether CCT is capable of improving this dysfunction and to explore the possible mechanisms. Endothelial dysfunction was induced by in vivo feeding high cholesterol diet (HCD) to rabbit and by in vitro treating bovine aortic endothelial cells (BAECs) with oxidized LDL (oxLDL). Endothelium-dependent relaxation (EDR) evoked by acetylcholine (Ach) and endothelium-independent relaxation (RIDR) mediated by sodium nitroprusside (SNP) of thoracic aorta isolated from rabbit were measured. The results indicated that the EDR in HCD alone treated rabbits was seriously impaired and the maximal relaxation induced by Ach ($10^{-5.5}$ M) was only 54% that in control rabbit fed with regular diet. Oral complementation with CCT (15, 30 mg/kg) dose-dependently improved this impairment and restored the maximal relaxation to 68% and 80% that in control group, respectively. However, the EIDR maintained comparable in all groups. Complementation with CCT (15, 30 mg/kg) simultaneously increased serum level of nitric oxide (NO), upregulated vessel activity and mRNA expression of endothelial NO synthase (eNOS) as well as vessel cyclic GMP (cGMP) content compared with those in rabbit treated with HCD alone. Inducible NOS (iNOS) activity remained unchangeable in all groups. In BAECs, oxLDL treatment decreased NO production, downregulated both activity and mRNA expression of eNOS. While those decrease or downregulation were inhibited by co-treatment with CCT (0.1, 1, 10 μM) in a dose-dependent manner. These findings suggested that CCT significantly restored the EDR of thoracic aorta in hypercholesterolemic rabbit, which might be explained by its action to increase the vessel eNOS activity, leading to elevation of NO production.

1. Introduction

The vascular endothelium plays an important role in the control of blood flow and thus tissue oxygenation by means of the release of nitric oxide (NO) [1]. Consequently, an impairment of this endothelial function appears to play a key role in several cardiovascular diseases, particularly in atherosclerosis (AS) [2,3]. The impairment already occurs at an early state of the disease [4], preceding macroscopically visible lesions

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which are characteristic of advanced states [5]. Unlike the vascular lesions, defects of endothelial function can be detected not only in conduit arteries but also in resistance vessel [6]. Earlier studies [7,8] confirmed that while receptor-mediated endothelium-dependent relaxation (EDR) to acetylcholine (Ach) as well as receptor-independent responses to calcium ionophore A23187 was lost, endothelium-independent agents such as the NOs donor sodium nitroprusside (SNP) elicited normal vasodilation in thoracic aorta of hypercholesterolemic rabbit. These observations implied the site of inhibition of EDR to be distal to receptor-mediated events and proximal to the activation of the vascular smooth muscle, suggesting that impaired enzymatic synthesis of NOs could be one of the mechanisms causing endothelial dysfunction. NO is produced by an enzyme known as NO synthase (NOS) which converts L-arginine to L-citrulline and NO [9,10]. There are two isoforms of NOS in endothelial cells, endothelial NOS (eNOS) and inducible NOS (iNOS). NO originated from eNOS is thought to play a pivotal role in maintaining the vasorelaxation. Ooboshi et al. [11] reported that overexpression of eNOS gene in AS animals improved the EDR by increasing NO production. Otherwise, chronical administration of NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) to rabbits resulted in reduced NO production and completely abolished the EDR evoked by Ach [12]. Therefore, alleviation of endothelial dysfunction by increasing NO production would be beneficial to prevention and treatment of AS. It is likely that oxidized low density lipoproteins (oxLDL) which accumulate in the arterial wall in hypercholesterolemia subjects play a causal role in the reduction of NO production through downregulation of eNOS mRNA expression [13,14], suggesting a possible mechanism by which oxLDL contributes to impairment of EDR and consequently to AS plaque formation.

Crocetin (CCT), a carotenoid extracted from *Gardenia jasminoides* Ellis, is widely used for the prevention and treatment of cardiovascular diseases, largely depending on its anti-oxidation effect [15]. We have previously reported that CCT exerted the anti-AS action on animal models of both rat and rabbit [16,17]. The mechanism exploration showed that the inhibition of oxLDL production and consequently the alleviation of injury to vascular cells caused by oxLDL might be partly responsible for the anti-AS action of CCT [18]. Because endothelial dysfunction strongly contributes to the initiation and progression of atherosclerosis, the present study aims to investigate whether CCT is capable of improving this dysfunction of thoracic aorta isolated from hypercholesterolemic rabbit and to explore the possible mechanisms.

2. Materials and methods

2.1. Drugs and chemicals

Crocetin (CCT) was extracted from *G. jasminoides* Ellis in our laboratory and its purity is over 98% (assayed by high performance liquid chromatography, HPLC) (chemical structure shown as Fig. 1). Phenylephrine (PE), acetylcholine hydrochloride (Ach), sodium nitroprusside (SNP), indomethacin were from Sigma (USA). DMEM culture medium was from Gibico Company. Newly born bovine serum (NBS) was provided by Shanghai Weike Biochemical Reagent Co. Ltd. (China). Low density lipoprotein (LDL) was purchased from Nanjing Military Hospital (China). Kits for determinations of nitric oxide (NO), endothelial NO synthase (eNOS), inducible NOS (iNOS) and protein were provided by Nanjing Jiancheng Bioengineering Company (China). Kit for cyclic guanine monophosphate (cGMP) was the product of TCM University (China). Tripul was the product of Roche Company. dNTP, Moloney murine leukemia virustranscriptase (MMLV), Taq DNA polymerase, RNAsin, oligo(dT)15 primer and oligonucleotides for eNOS (both rabbit and bovine) and GAPDH were from Sangong Biotechnology (Shanghai, China). The following primer pairs were used—rabbit eNOS: sense, 5′-GCT GCG CCA GGC TCT CAC CTT C-3′; antisense, 5′-GGC TGC AGC CCT TTG CTC TCA A-3′; BAECs eNOS: sense, 5′-GAG CCA CAG AGC AGA CGG AG-3′; antisense, 5′-CAC TCT CTC GGA GGT GGA TG-3′; GAPDH: sense, 5′-ATC ACC ATC TTC CAG GAG CG-3′; antisense, 5′-CCT GCT TCA CCA CCT TCT TG-3′. Other chemicals used were analytical grade.

2.2. Animal preparations

Male New Zealand rabbits weighing 2.0–2.5 kg were provided by Animal Experimental Center of China, Pharmaceutical University (China) and all experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China (1998). The rabbits were randomly divided into four groups of eight animals each: control group, HCD group, HCD + CCT (30) group and HCD + CCT (15) group. Except for the rabbits in control group fed with regular diet, those in other groups were fed with high cholesterol diet (HCD) containing regular diet (94.8%), lard (4%) and cholesterol (1.2%) for 8 weeks. In the CCT groups, the diet was prepared daily by mixing CCT (30 and 15 mg/kg body weight), respectively, into 20 g HCD and by checking that rabbits completely consumed their food. All animals received 120 g of food daily. All animals were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and sacrificed by exsanguinations at the end of experiment.

2.3. Arterial rings preparation and protocol

At the end of the experiment, rabbits were anaesthetized with sodium pentobarbital and sacrificed by exsanguinations from abdominal aorta. The thoracic aorta isolated from rabbits was placed in ice cold Krebs solution (mM) (NaCl 118.3; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO4 1.2; glucose 11.1 and $NaHCO_3$ 24.9, pH 7.4), cleaned from connective tissues and cut into transverse rings of 3 mm long. Special care was taken to avoid damage to the endothelium. Each ring was then suspended vertically in the organ chamber (volume 20 ml) between two

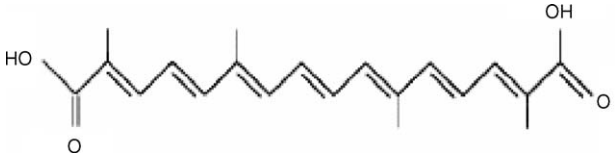

Fig. 1 – Chemical structure of CCT.