**Ginkgo biloba** extract (GbE) enhances the anti-atherogenic effect of cilostazol by inhibiting ROS generation

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http://dx.doi.org/10.3858/emm.2012.44.5.035

Accepted 18 January 2012
Available Online 27 January 2012

Abbreviations: ApoE, apolipoprotein E; cilostazol, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone; GbE, Ginkgo biloba extract; MCP-1, monocyte chemoattractant protein-1 (MCP-1); sVCAM-1, soluble vascular cell adhesion molecule-1 (sVCAM-1); VCAM-1, vascular cell adhesion molecule-1

Abstract

In this study, the synergistic effect of 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone (cilostazol) and **Ginkgo biloba** extract (GbE) was examined in apolipoprotein E (ApoE) null mice. Co-treatment with GbE and cilostazol synergistically decreased reactive oxygen species (ROS) production in ApoE null mice fed a high-fat diet. Co-treatment resulted in a significantly decreased atherosclerotic lesion area compared to untreated ApoE mice. The inflammatory cytokines and adhesion molecules such as monocyte chemoattractant-1 (MCP-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), and VCAM-1 which can initiate atherosclerosis were significantly reduced by the co-treatment of cilostazol with GbE. Further, the infiltration of macrophages into the intima was decreased by co-treatment. These results suggest that co-treatment of GbE with cilostazol has a more potent anti-atherosclerotic effect than treatment with cilostazol alone in hyperlipidemic ApoE null mice and could be a valuable therapeutic strategy for the treatment of atherosclerosis.

Keywords: atherosclerosis; cilostazol; cytokines; disease models, animal; **Ginkgo biloba**; inflammation; macrophages; reactive oxygen species

Introduction

Atherosclerosis is a chronic inflammatory disease of blood vessels characterized by slow thickening of arterial walls due to the build-up of fatty material (Chen et al., 2003; Park et al., 2008). During the early stages of atherosclerosis, cholesterol accumulation in the intima induces endothelial cells in the arteries to express adhesion and chemoattractant molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) (Otsuki et al., 2001; Lee et al., 2005; Yun et al., 2009). Reactive oxygen species (ROS), including superoxide, are implicated in the cellular response to a variety of inflammatory stimuli, including atherosclerosis (Zhou et al., 2000; Altiok et al., 2006; Rhein et al., 2010). 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone (cilostazol) is a selective phosphodiesterase III inhibitor that increases the intracellular cyclic adenosine monophosphate (cAMP) concentration (Kim et al., 2002, 2006; Lim et al., 2009). Cilostazol inhibits cytokine-induced nuclear factor-κB (NF-κB) activation via AMP-activated protein kinase activation in vascular endothelial cells (Nakamura et al., 2005; Hattori et al., 2009). Besides anti-platelet and anti-vasoconstrictive properties (Wang et al., 2003; Mohamed, 2009), cilostazol promotes cholesterol efflux by regulating...
cholesterol uptake- or efflux-related genes, such as scavenger receptors (e.g., SR-A and CD36) (Shin et al., 2004; Gomez and Qureshi, 2009) and ABCA1/ABCG1 (Nakaya et al., 2010) in macrophages. Cilostazol inhibits NAD(P)H oxidase-dependent superoxide formation and cytokine release concurrent with the suppression of atherosclerotic plaque formation in LDL receptor-null mice (Yun et al., 2009).

Ginkgo biloba extract (GbE), a Chinese herbal medicine extracted from leaves of the Ginkgo biloba tree (Chen et al., 2003), has increasingly been shown to have a variety of beneficial effects in cerebral and peripheral arterial diseases, especially dementia and claudication (Wei et al., 1999; Lee et al., 2001; Wang et al., 2003; Sethi and Arora, 2008). GbE contains flavone glycosides and 6% terpene lactones (ginkolides, bilobalide), known free radical scavengers (Kampkotter et al., 2007; Ou et al., 2009). GbE also exerts an anti-phlogistic effect on inflammatory cells by suppressing active oxygen and nitrogen species production (Ou et al., 2009). For example, the terpene lactone component in GbE inhibits nitric oxide (NO) production in macrophages infiltrating a Candida albicans-mediated arthritic inflammation site (Lippi et al., 2007).

Recently, GbE was shown to reduce the formation of atherosclerotic nanoplaques (Rodriguez et al., 2007), attenuate oxLDL-induced oxidative functional damage in endothelial cells (Ou et al., 2009), and decrease the levels of highly atherogenic lipoprotein (Lippi et al., 2007; Rodriguez et al., 2007; Siegel et al., 2007). Thus, GbE may at least partially have an anti-inflammatory effect, and supplementation with GbE may have clinical value in patients at risk for increased serum concentrations of lipoprotein (Lippi et al., 2007).

The combination of cilostazol and probucol, another potent lipid-soluble antioxidant, displayed a synergistic effect on the suppression of ROS and inflammatory markers in human coronary artery endothelial cells (Park et al., 2008). Moreover, GbE may potentiate the anti-platelet effect of cilostazol without prolonging bleeding or coagulation times (Ryu et al., 2009). Although the anti-atherogenic effects of both cilostazol and GbE have been suggested in previous studies, the synergistic effect of these two compounds on atherosclerosis has not been investigated.

Here, we show that combination therapy consisting of cilostazol and GbE may exert enhanced anti-atherogenic effects compared to treatment with cilostazol alone.

Results

GbE increases the anti-oxidant activity of cilostazol

Both cilostazol and GbE reduce ROS production in a variety of cell types (Wei et al., 1999; Kim et al., 2002; Kampkotter et al., 2007) and have a synergistic effects in treating atherothrombosis without adverse side effects such as the prolongation of bleeding time or coagulation time (Liu et al., 2009). Therefore, we postulated that combinative treatment of an atherosclerotic mouse model with GbE and cilostazol would decrease superoxide production in atherosclerotic plaque more than treatment with cilostazol alone. Superoxide production in the plaque lesion of the aortic root was decreased in all the treated groups, and also was lower in the high dose co-treatment group than cilostazol alone (Figure 1). This suggests that co-treatment of cilostazol with GbE synergistically inhibits ROS production in the development of atherosclerosis.

![Figure 1. GbE increases the anti-oxidant activity of cilostazol. DHE fluorescence image of aortic root area from vehicle (n = 5), 0.1% cilostazol (n = 9), 0.05% cilostazol + 0.04% GbE (n = 9) and 0.1% cilostazol + 0.08% GbE treated groups (n = 12 each). Quantitative data in the lower graph represent arbitrary units for fluorescence intensity. L, lumen. Yellow arrows indicate superoxide-positive areas. Scale bars, 200 μm. **P < 0.01 and ***P < 0.001 compared with vehicle; and ###P < 0.001 compared with cilostazol alone.](image-url)
GbE synergistically increases the anti-atherogenic effect of cilostazol

To determine how the anti-oxygenic effect of these two compounds affects the development of atherosclerosis, we analyzed atherosclerotic lesions in ApoE null mice fed a high-fat diet for 16 weeks. Sections of the aortic root from untreated mice showed a large plaque lesion area in the vessel walls. As expected, mice treated with cilostazol (0.1%) and GbE (0.08%) showed a significant reduction in the size of the atherosclerotic lesion in the aortic root (0.48 ± 0.06 mm² vs 0.56 ± 0.05 mm² in 0.1% cilostazol, 0.08% GbE treatment group and vehicle treatment group, respectively; *P < 0.05 compared with vehicle (Fig 2A). Plaque area in the aortic arch and descending aorta was also reduced in mice treated with cilostazol (0.1%) and GbE (0.08%) compared with control mice (9.26 ± 0.57% vs 11.78 ± 2.5% in 0.1% cilostazol, 0.08% GbE treatment group and vehicle treatment group, respectively; *P = 0.05; Fig 2B). Total cholesterol and triglyceride levels in serum were significantly decreased in mice treated with 0.1% cilostazol alone, however co-treatment of cilostazol and GbE showed no significant changes (data not shown).

Co-treatment with cilostazol and GbE decreases pro-inflammatory cytokine production

Next, we investigated whether these two compounds can affect the production of pro-inflammatory molecules in blood. The monocyte chemoattractant-1 (MCP-1) level was significantly decreased in mice treated with cilostazol alone and also in those co-treated with a high dose of cilostazol and GbE. The expression level of soluble vascular cell adhesion molecule (sVCAM-1) was significantly decreased in the co-treatment group. However, interleukin-6 (IL-6) levels were not changed in the co-treatment group (Table 1). To confirm the changes of these molecule expressions in the plaque area, we performed immunohistochemistry. Compared with the control group, co-treatment of cilostazol with GbE decreased the expression of MCP-1 (Figure 3A) and VCAM-1 (Figure 3B).

Co-treatment with cilostazol and GbE inhibits macrophage infiltration

We measured infiltrated macrophages in the atherosclerotic plaque area in order to determine if the production of MCP-1 and VCAM-1 lead to a decrease in macrophage infiltration into the aortic intima. Macrophage infiltration was lower in the high dose co-treatment group than cilostazol alone. These data suggest that co-treatment of cilostazol with GbE exerts a synergistic effect on the inhibition of macrophage infiltration into the arterial walls (Figure 4).

Discussion

In this study, we show that co-treatment of cilostazol
with GbE reduces superoxide production following decreased atherosclerotic plaque formation. Co-treatment of cilostazol with GbE also lowered sVCAM-1 and MCP-1 levels in serum, and reduced macrophage infiltration into the aortic intima. Our observations indicate that cilostazol and GbE exert synergistic anti-atherosclerotic effects. Indeed, we have demonstrated that co-treatment of cilostazol with GbE induced a reduction in atherosclerotic lesion.

Increased ROS generation such as superoxide may be involved in the development of atherosclerosis (Dandona et al., 2010). ROS-dependent mechanisms can increase the expression of adhesion molecule such as VCAM-1, leading to inflammatory cell recruitment and infiltration into the intima region (Chen et al., 2003; Lee et al., 2005; Ou et al., 2009). In atherosclerotic conditions, treatment with either cilostazol or GbE markedly attenuates ROS production by a distinct mechanism. Cilostazol blocks ROS production via inhibition of NADPH oxidase (Shin et al., 2004; Yun et al., 2009). It also reduces CD36 or SR-A expression in murine macrophages via inhibition of NADPH oxidase-derived ROS production, which leads to reduced foam cell formation (Okutsu et al., 2009;
Figure 4. Co-treatment of cilostazol and GbE inhibits macrophage infiltration. Representative immunostaining for macrophages in the aortic root area from vehicle (n = 5), 0.1% cilostazol (n = 10), 0.05% cilostazol + 0.04% GbE (n = 8), and 0.1% cilostazol + 0.08% GbE treated groups (n = 8). Quantitative data in the lower graph represent positive stained area percentage of total plaque area. L, lumen. Yellow arrows indicate a macrophage-positive area. Scale bars, 200 μm. **P < 0.01 compared with vehicle; #P < 0.05 compared with cilostazol alone.

Yun et al., 2009). A recent study also showed that cilostazol inhibited oxidative stress and subsequent cellular senescence by enhancement of NO production in HUVECs. Cilostazol can induce NO production via a cAMP/PKA- and PI3K/Akt-dependent mechanism, thereby delaying endothelial cellular senescence. Cellular senescence of endothelial cells has been proposed to be involved in endothelial dysfunction and atherosclerosis (Ota et al., 2008).

Inflammation is involved in the initiation, rupture, and thrombosis of atherosclerotic plaques (Lee et al., 2005). Some studies have suggested that cilostazol and GbE have anti-inflammatory effects (Lippi et al., 2007; Mohamed, 2009; Aoki et al., 2010). GbE contains high levels of terpene, and this biflavonoid decreases the levels of IL-6, IL-8, and tumor necrosis factor (TNF)-alpha through the down-regulation of NF-κB DNA binding activity in patients with pulmonary interstitial fibrosis (Lippi et al., 2007). Previous studies have reported that cAMP selectively suppresses expression of VCAM-1 and endothelial leukocyte adhesion molecule-1 (ELAM-1) (Pober et al., 1993). Moreover, VCAM-1 plays a major role in the initiation of atherosclerosis (Cybulsky et al., 2001). Given the role of cilostazol as a cAMP activator, these previous findings are in agreement with our results. In addition, MCP-1 is a crucial factor for the development of atherosclerosis. Whereas VCAM-1 exerts a dominant role in the initiation of atherosclerosis, increased MCP-1 expression was demonstrated to mediate chronic inflammation. Both preferentially contribute to monocyte adhesion (Lee et al., 2005; Choi et al., 2011). We show that elevated macrophage infiltration is accompanied by high expression of VCAM-1 and MCP-1 in serum and the atherosclerotic plaque region. Although MCP-1 levels in serum appear to be mainly affected by cilostazol in our study, the level of MCP-1 in atherosclerotic plaque was decreased by co-treatment with cilostazol and GbE, but not cilostazol alone. These findings all show that atherosclerosis is significantly reduced by co-treatment with cilostazol and GbE compared to treatment with cilostazol alone.

Taken together, the our data support the hypothesis that the anti-atherosclerotic effect of cilostazol and GbE can be attributed to reduced superoxide generation, macrophage infiltration, and expression of pro-inflammatory molecules such as VCAM-1 and MCP-1. The major finding of the present study is that co-treatment of cilostazol with GbE significantly decreased atherosclerotic plaque in the aorta of ApoE null mice fed a high-fat diet, compared to treatment with cilostazol alone. In conclusion, we show that combinative therapy of cilostazol with GbE might exert an enhanced anti-atherogenic effect compared to treatment with cilostazol alone.

Methods

Animals and diets

ApoE null (C57BL/6J background) male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated to the facility for at least 2 weeks before beginning the experiments. Mice were housed five to six per cage and maintained on a 12-h light/12-h dark cycle with water ad libitum. Eight-week-old male ApoE null mice were randomly divided into five groups including: normal chow (n = 5), vehicle (n = 11), cilostazol (n = 10) and both co-treatment groups (n = 12 per group). The animals were fed a high-fat diet (20% fat, 0.15% cholesterol, Research Diets, New Brunswick, NJ) supplemented with 0.1% cilostazol, or both 0.05% cilostazol and 0.04% GbE, or both 0.1% cilostazol and 0.08% GbE for test groups (0.1% lactose for vehicle group) for 16 weeks respectively. Control...
mice were fed ordinary normal chow diet (PMI Nutrition International, LLC Certified Rodent LABDIET, Purina Mills, Richmond, IN). Body weights were monitored every week. All animal study protocols were approved by the Institutional Animal Care and Usage Committee of the Ewha Womans University (Seoul, Korea).

Genotyping
Genotyping was performed to confirm ApoE deficiency. Genomic DNA was extracted from mouse tails. For PCR of ApoE, the forward and reverse primers for the wild type allele were 5′-AGAACTGACGTAGTGTC3′-3′ and 5′-GGTCCAGAAGTTGAAAGC-3′ (expected product -300 bp), respectively. For the null allele, the same forward primer was used and the reverse primer was 5′-GCTTCCCTCGTCTTACGTA-3′ (expected product -200 bp). PCR was carried out with all three primers in the same reaction mix. PCR conditions were: 94°C, 45 s; 58°C, 45 s; and 72°C, 45 s for 30 cycles.

Atherosclerosis quantification
After mice were euthanized, hearts and aortas were perfused with phosphate-buffered saline (PBS) through the left ventricle. The aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and adventitial fat was removed. Aortas were opened longitudinally, these were pinned onto a flat black silicone plate with 2 cm needles. The hearts and pinned aortas were fixed with 10% neutral buffered formalin solution for 16 h. For lesion quantification in the aortic root, the hearts were removed at the proximal aorta and the upper portion was embedded in OCT compound (Tissue-Tek) and frozen at -70°C. Ventricular tissue was sectioned into 10 μm sections by a cryostat microtome (Leica CM18050 XL). Sections and fixed aortas were immersed in absolute propylene glycol (Duchefa Biochemie) for 5 min at RT. Fixed tissue was dehydrated, antigen retrieval was carried out with PBS + 0.1% Triton X-100 for 30 min, images were observed using a fluorescence microscope (Axiovert 200 Basic Stand, Carl Zeiss, Inc.). The quantitative analysis is expressed as a percentage of DHE-stained area per total lesion area in the aortic root using Axiovision AC (Carl Zeiss, Inc.).

Bacterial culture and cytokine analysis
Blood was collected from the retro-orbital sinus into non-heparinized capillary tubes (Scientific Glass, Inc). Thereafter serum was obtained by centrifugation at 13,000 g for 10 min at 4°C and stored at -70°C before analysis. Total cholesterol, triglyceride, HDL, and LDL cholesterol levels were measured. To quantify cytokines in serum, MCP-1 and sVCAM-1 levels were estimated using ELISA kits (R&D Systems).

Measurement of superoxide in situ
The frozen sections of aortic root in the slide were dried for 2 h at 37°C and washed with distilled water for 5 min. The samples were incubated to expose antigen with PBS + 0.1% Triton X-100 (Juncei Chemical Co., Ltd.) for 15 min and then incubated with 5 μM dihydroethidium (Molecular Probes, Eugene, OR) in a light-shielded state to estimate superoxide levels. The washing step was performed with PBS + 0.1% Triton X-100 buffer at least three times for 5 min per wash. After treatment of DAPI solution (Sigma Aldrich) for 30 min, images were observed using a fluorescence microscope (Axiovert 200 Basic Stand, Carl Zeiss, Inc.). The quantitative analysis is expressed as a percentage of DHE-stained area per total lesion area in the aortic root using Axiovision AC (Carl Zeiss, Inc.).

Statistical analysis
Statistical significance was determined by the Student’s t-test and Mann-Whitney U Test. A value of P < 0.05 was considered significant.

Acknowledgements
We thank SK chemicals for supporting this study. This study was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare (A090264), Korea.

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