Ginkgolide A attenuates homocysteine-induced endothelial dysfunction in porcine coronary arteries

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Background: Homocysteine is an independent risk factor for atherosclerosis. The objective of this study was to investigate whether ginkgolide A (GA), a major constituent of *Ginkgo biloba*, could block homocysteine-induced endothelial dysfunction in porcine coronary arteries.

Methods: Porcine coronary artery rings were assigned to six treatment groups: control; homocysteine (50 μmol/L); low-dose (50 μmol/L) or high-dose (100 μmol/L) GA; and homocysteine plus low-dose or high-dose GA. After 24 hours' incubation, the rings were analyzed for vasomotor function in response to a thromboxane A2 analogue (U46619), bradykinin, and sodium nitroprusside. Endothelial nitric oxide synthase (eNOS) was studied by using real-time polymerase chain reaction and immunohistochemistry analysis. Superoxide anion production was assessed by chemoluminescence analysis.

Results: Endothelium-dependent relaxation (bradykinin) was significantly reduced in ring segments treated with homocysteine as compared with the control ($P < .05$). When homocysteine was combined with either low-dose or high-dose GA, endothelium-dependent relaxation was markedly recovered. There was no significant difference in maximal contraction (U46619) or endothelium-independent relaxation (sodium nitroprusside) among all groups. In addition, superoxide anion production was increased by 113% in the homocysteine-treated group, whereas there was no statistically significant difference between the control and GA/homocysteine groups. Furthermore, eNOS messenger RNA and protein levels were substantially reduced in the homocysteine-treated group ($P < .05$), but not in the GA/homocysteine combined groups.

Conclusions: Homocysteine significantly impairs endothelium-dependent vasorelaxation through oxidative stress and downregulation of eNOS in porcine coronary arteries. GA effectively prevents homocysteine-induced endothelial dysfunction and molecular changes in porcine coronary arteries. This study underscores the potential clinical benefits and applications of GA in controlling homocysteine-associated vascular injury and cardiovascular disease. (J Vasc Surg 2006; 44:853-62.)

Clinical Relevance: Homocysteine is an independent risk factor for atherosclerosis. This study showed that ginkgolide A, a major constituent of *Ginkgo biloba,* effectively prevents homocysteine-induced endothelial dysfunction and molecular changes in porcine coronary arteries. This study underscores potential clinical benefits and applications of ginkgolide A in controlling homocysteine-associated vascular injury and cardiovascular disease.

It is widely accepted that endothelium dysfunction is an initial step of atherosclerosis by altering endothelium dependent factors, modifying blood vessel permeability, and changing platelet and leukocytes adhesions. Homocysteine is an independent risk factor for atherosclerotic diseases. Studies have shown that plasma homocysteine levels correlate with cardiovascular mortality in a concentration-dependent fashion and that the relationship is strongest when total plasma homocysteine levels are more than 15 μmol/L.[1,2] The basis for these results has been extensively investigated, yet it is not fully elucidated. A growing body of evidence supports that oxidative stress plays an important role in the effects of homocysteine on the cardiovascular system.[3] Our earlier study also demonstrated that homocysteine significantly impaired endothelium-dependent vasorelaxation, decreased endothelial nitric oxide synthase (eNOS) immunoreactivity, and increased superoxide anion production in porcine coronary arteries.[4-6] Furthermore, our previous studies showed that antioxidant-based pharmacotherapy can effectively reduce homocysteine-induced endothelial dysfunction and free radical production.[6-9] These studies imply an important future therapeutic strategy of using antioxidant-based pharmacotherapy for counteracting the detrimental effects of hyperhomocysteinemia.

Ginkgo biloba, one of the oldest living tree species, referred to as "a living fossil" by Charles Darwin, is widespread throughout the world. The extract from its leaves is one of the most widely used herbal supplements and has

become increasingly popular in recent years. Several studies have shown the potential benefits of *Ginkgo biloba* in cardiovascular diseases through its protective roles against free radical injury on the vascular endothelium.[10-13] However, to our knowledge, there are no studies of these extracts in hyperhomocysteinemic patients, who often have endothelial dysfunction. We therefore chose to study the effect of ginkgolide A (GA) on the vascular endothelium—particularly the potential effects on homocysteine-induced injuries. This is a novel investigation. We hypothesized that *Ginkgo biloba* and its active constituent, GA, could protect the endothelium from homocysteine damage. Specifically, we studied the effects of homocysteine and GA on vasomotor function, eNOS expression, and superoxide production in porcine coronary arteries. This study may suggest a potential clinical application of *Ginkgo biloba* in controlling homocysteine-associated vascular injuries.

MATERIALS AND METHODS

Chemicals and reagents. DL-Homocysteine, dimethyl sulfoxide (DMSO), 9,11-dideoxy-11a, 9a-epoxymethanoprostaglandin F_{2a} (U46619), bradykinin, *Ginkgo biloba* A (GA), Tri-Reagent, Tris-buffered saline solution, and phosphate-buffered saline (PBS) solution were obtained from Sigma (St Louis, Mo). Dulbecco modified Eagle medium was obtained from Life Technologies, Inc (Grand Island, NY). Porcine coronary artery endothelial cell (PCAEC) growth medium was purchased from Cell Applications Inc (San Diego, Calif). Antibiotic-antimycotic was obtained from Mediatech Inc (Herndon, Va). The iScript cDNA Synthesis Kit and iQ SYBR Green SuperMix Kit were obtained from Bio-Rad Laboratories (Hercules, Calif). Antibody against human eNOS was obtained from BD Transduction Laboratories (Lexington, Ky). The biotinylated horse anti-mouse immunoglobulin G and avidin-biotin complex kit were obtained from Vector Labs (Burlingame, Calif).

Myograph analysis. The myograph system used in our laboratory has been previously described.[6-9] Fresh porcine hearts were harvested from young adult farm pigs (6-7 months old) at a local slaughterhouse, placed in a container filled with cold PBS solution, and immediately transported to the laboratory. The pig right coronary arteries were carefully dissected and cut into multiple 5-mm rings. The rings were then incubated in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, Calif) with different concentrations of GA and homocysteine (50 µmol/L) at 37°C with 5% carbon dioxide in a cell-culture incubator for 24 hours. The concentrations used were GA at 25, 50, and 100 µmol/L. After the pilot study, the concentrations of GA at 50 and 100 µmol/L were determined for the remaining studies. The right coronary artery rings were divided into six groups (eight rings per group): DMSO control (solvent control), homocysteine alone (50 µmol/L), low-dose (50 µmol/L) or high-dose (100 µmol/L) GA alone, and homocysteine plus low-dose or high-dose GA. After incubation, the artery rings were analyzed by myograph analysis (Danish Myo Technology Organ Bath 700 MO,

Aarhus, Denmark). Each ring was treated with a thromboxane A2 analogue (U46619; 10^{-7} mol/L) to generate maximal contraction. The relaxation dose-response curve was generated by adding 60 µL of five cumulative doses of the endothelium-dependent vasodilator bradykinin (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} mol/L) at 3-minute intervals. Subsequently, endothelium-independent relaxation was induced by adding sodium nitroprusside (10^{-5} mol/L). Contractility and percentage of relaxation were calculated according to the tension changes. The data of the coronary artery rings from eight different pig hearts were averaged and represented as one data point for statistical analysis.

Cell culture. PCAECs and PCAEC growth medium were purchased from Cell Applications Inc. Cells were used at passages 4 to 6. When PCAECs grew to 80% to 90% confluence in six-well plates, they were treated with homocysteine (50 µmol/L) with or without different concentrations (1, 10, 20, 50, or 100 µmol/L) of GA for 24 hours. In all groups, cells cultured in DMSO (0.5% vol/vol) alone were used as solvent controls.

Real-time polymerase chain reaction. Total RNA from culture PCAECs or porcine artery endothelial cells was isolated by using Tri-Reagent according to the manufacturer's instructions. Complementary DNA was generated by reverse transcription from messenger RNA (mRNA) by using the iScript cDNA Synthesis Kit according to the manufacturer's instructions. Then the iQ SYBR Green SuperMix Kit was used for real-time polymerase chain reaction (PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control for eNOS expression, to account for variations in mRNA loading. The same total RNA, 1 µg, was loaded for all samples. Porcine eNOS and GAPDH primers were designed by using Beacon Designer (Primer Biosoft Inc, Palo Alto, Calif). The eNOS primer sequences were as follows: forward primer, 5′-CCCTACAACG-GCTCCCCTC-3′; reverse primer, 5′-GCTGTCTGTGT-TACTGGATTCCTT-3′. The GAPDH primer sequences were as follows: forward primer, 5′-TGTACCACCAACT-GCTTGGC-3′; reverse primer, 5′-GGCATGGACTGTG-GTCATGAG-3′. Real time PCR was performed in an iCycler iQ real-time PCR detection system. The thermal cycle condition used for reverse transcription was as follows: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. The conditions used for real-time PCR were as follows: 3 minutes at 95°C, 40 repeats of 20 seconds at 95°C, and 1 minute at 60°C. Controls were performed with no reverse transcription (mRNA sample) or water control for both eNOS and GAPDH to demonstrate the specificity of the primers and the lack of DNA contamination in samples. Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units vs PCR cycle numbers during exponential amplification so that sample measurement comparisons were possible. The relative expression for eNOS in each sample was normalized against GAPDH, shown as $2^{[Ct(GAPDH) - Ct(eNOS)]}$.

Immunohistochemistry. Rings of treated porcine coronary arteries were fixed overnight in 10% neutral buff-

ered formalin and subsequently stored in 70% alcohol until processing. Samples were later embedded in paraffin, cut into 5-μm-thick cross sections, and mounted onto slides. Sections were treated with 3% H_2O_2 in PBS (pH 7.4) for 10 minutes to quench the endogenous peroxidase activity. After three 10-minute rinses in 0.1 mol/L PBS, sections were incubated in monoclonal antibody against human eNOS (1:1000) diluted in 0.1 mol/L PBS containing 5% normal horse serum and 0.1% Triton X-100 overnight at 4°C. After another three 10-minute rinses in 0.1 mol/L PBS, sections were incubated with biotinylated anti-mouse immunoglobulin G (1:250) at room temperature for 40 minutes. For diaminobenzidine visualization, sections were incubated in avidin-biotin-peroxidase solution at room temperature for 1 hour, followed by 0.1% diaminobenzidine and 0.003% H_2O_2 in Tris-buffered saline for 5 to 10 minutes. Sections were counterstained with hematoxylin and eosin, coverslipped, and viewed on an Olympus BX41 microscope (Olympus USA, Inc, Melville, NY). Images were captured with an attached SPOT-RT digital camera and software (Diagnostic Instruments Inc, Sterling Heights, Mich).

Lucigenin-enhanced chemiluminescence analysis. Levels of superoxide anion produced by endothelial cells were detected by using the lucigenin-enhanced chemiluminescence method with a Sirius Luminometer and FB12 software from Berthold Detection System GmbH (Pforzheim, Germany). The rings were cut open longitudinally and trimmed into approximately 5 × 5-mm pieces. They were then rinsed briefly in a modified Krebs HEPES buffer solution (120 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L K_2HPO_4, 20 mmol/L HEPES, 2.5 mmol/L $CaCl_2$, 1.17 mmol/L $MgSO_4$, and 25 mmol/L $NaHCO_3$). An assay tube (12 × 75 mm) was filled with 500 μL of Krebs HEPES buffer solution and 25 μL of lucigenin. After gentle vortexing, the vessel segments were placed endothelium side down in the tubes. Time-based reading of the luminometer was recorded by FB12 software. The data in relative light units (RLU) per second for each sample were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding vessel samples. The area of each vessel segment was measured with a caliper and used to normalize the data for each sample. Final data are represented as RLU per second per square millimeter.

Nitric oxide staining and flow cytometry assay. PCAECs were harvested with 0.02% trypsin/ethylenediaminetetraacetic acid and adjusted to 1×10^6 cells per fluorescence-activated cell-sorter tube. 3-Amino, 4-aminomethyl-2', 7'-difluorofluorescein Diacetate (DAF-FM DA) (10 μmol/L) was added to the tubes and incubated at 37°C for 20 minutes. Final samples were collected in 500 μL of staining buffer and stored at 4°C. Samples were analyzed with a FACScan and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) within 24 hours of preparation. In each experiment, at least 10,000 events were analyzed.

Statistical analysis. Data from the different groups were analyzed by using an unpaired Student t test (two tailed; Minitab software, Sigma Breakthrough Technologies, Inc, San Marcos, Tex). In addition, analysis of variance was used to analyze the data of endothelium-dependent relaxation in response to bradykinin. A P value <.05 was considered statistically significant. Data are reported as mean ± SE.

RESULTS

GA blocks homocysteine-induced endothelial dysfunction in porcine coronary arteries. The pilot study using GA at three different concentrations (25, 50, and 100 μmol/L) showed that GA at 25 μmol/L had minimal improvement on homocysteine-induced effects, whereas homocysteine combined with GA at 50 and 100 μmol/L showed marked recovery of homocysteine-induced vasomotor dysfunction. Therefore, the concentrations of GA at 50 and 100 μmol/L were selected for further study. The arterial rings were divided into six treatment groups: DMSO control, homocysteine alone (50 μmol/L), low-dose GA (50 μmol/L), high-dose GA (100 μmol/L), homocysteine combined with low-dose GA, and homocysteine combined with high-dose GA. There was no substantial difference in maximum vessel contraction or endothelium-independent relaxation among all groups ($P > .05$; Fig 1, *A* and *D*). Conversely, endothelium-dependent vasorelaxation was significantly different between the homocysteine-treated group and the controls. As shown in Fig 1, *B*, precontracted arterial rings in the homocysteine-alone group showed marked reduction in endothelium-dependent vasorelaxation (0.12% ± 0.47%, 4.2% ± 1.34%, 15.2% ± 2.5%, 28.9% ± 3.6%, and 44.1% ± 3.3%) in response to each cumulative dose of bradykinin (10^{-9} to 10^{-5} mol/L) as compared with the controls. Furthermore, endothelium-dependent relaxation was significantly impaired in rings treated with homocysteine alone as compared with controls (bradykinin 10^{-5} mol/L; Fig 1, *C*), whereas neither high-dose nor low-dose GA alone affects endothelium-dependent relaxation. Both GA/homocysteine combined groups showed complete recovery of endothelium-dependent vasorelaxation.

GA blocks homocysteine-induced eNOS downregulation in porcine coronary arteries and PCAECs. The levels of eNOS mRNA expression in porcine coronary arteries were measured by using real-time PCR analysis. The effects of homocysteine and GA on mRNA expression in the treated arterial rings are demonstrated in Fig 2. The homocysteine-alone group showed a 65% reduction of the eNOS mRNA level as compared with controls ($P < .05$), whereas the low- and high-dose GA groups had a level of eNOS mRNA comparable to the control levels ($P > .05$). In addition, although this was not statistically significant, the low-dose GA/homocysteine combined group showed partial improvement in the eNOS mRNA level, whereas the high-dose GA/homocysteine combined group showed significant recovery in the eNOS mRNA level compared with the homocysteine-alone group ($P < .05$). In addition, we
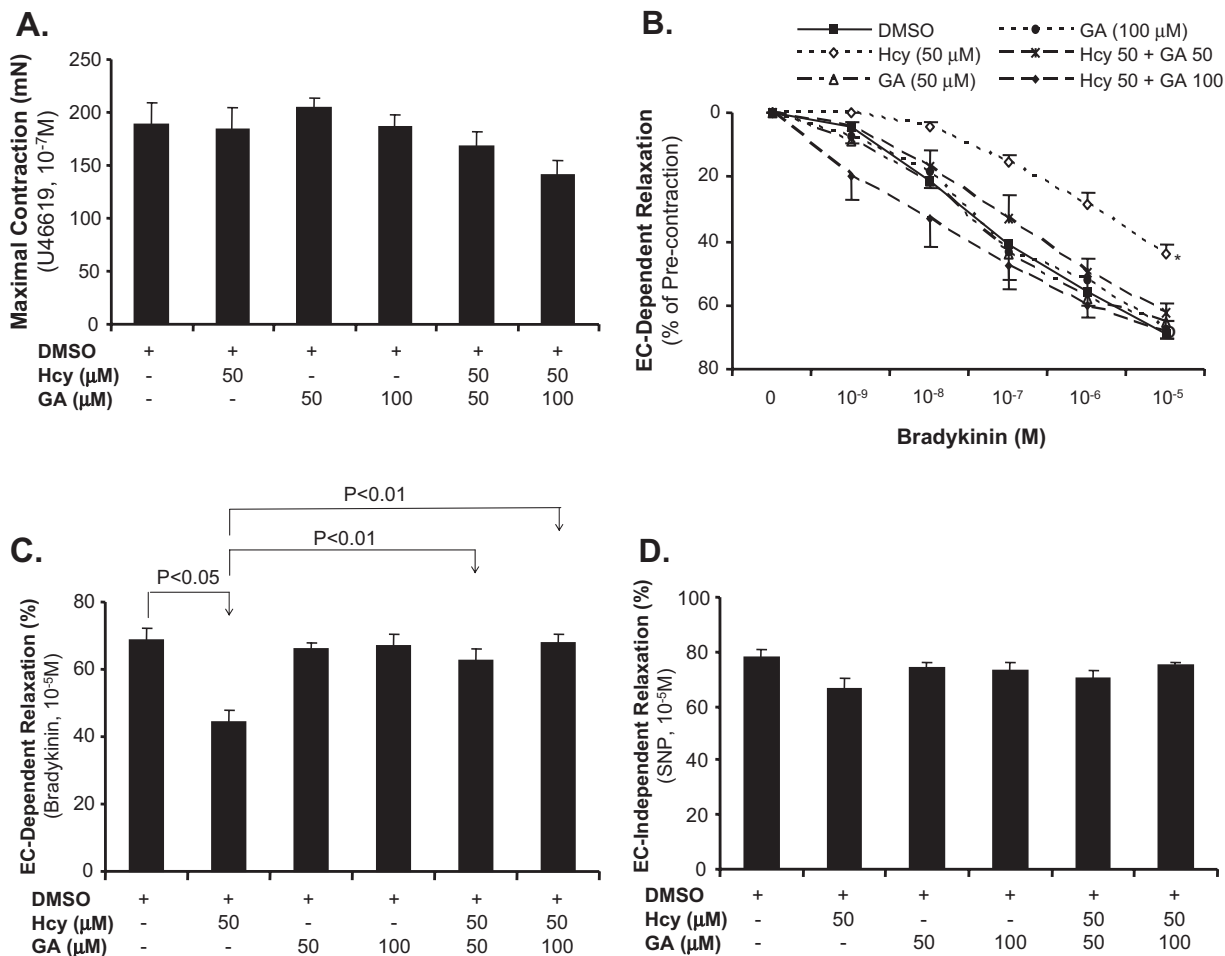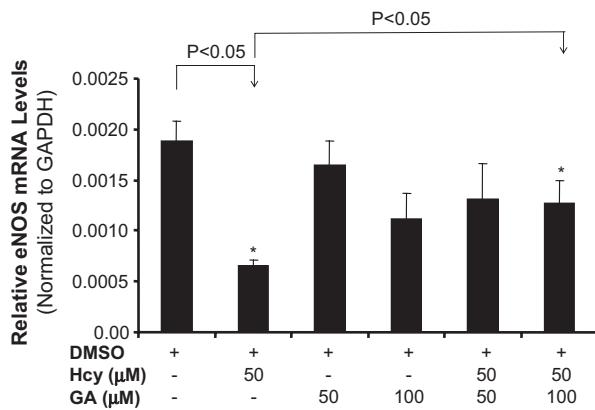
Fig 1. Effects of homocysteine *(Hcy)* and ginkgolide A *(GA)* on vasomotor function in porcine coronary arteries. **A,** Contractility. The rings (eight per group) were cultured in dimethyl sulfoxide *(DMSO)* control, homocysteine (50 μmol/L) alone, low-dose GA (50 μmol/L) alone, or high-dose GA (100 μmol/L) alone and homocysteine combined with low-dose or high-dose GA. There was no substantial difference in maximum vessel contraction in response to U46619 (10^{-7} mol/L) among all groups $(P > .05)$. **B,** Endothelium-dependent relaxation. Vasorelaxation in response to cumulative doses of bradykinin demonstrated improved vasorelaxation among all groups as the concentration of bradykinin increased. Both the low-dose and high-dose GA/homocysteine groups showed significant improvements in vasorelaxation compared with homocysteine alone at the bradykinin concentrations of 10^{-7}, 10^{-6}, and 10^{-5} mol/L. **C,** Vasorelaxation in response to the highest dose of bradykinin (10^{-5} mol/L). The homocysteine groups showed significant impairments in vasorelaxation as compared with the control $(P < .05)$, whereas both the low-dose and high-dose GA/homocysteine groups demonstrated a significant improvement in vasorelaxation as compared with homocysteine $(P < .05)$ and were similar to the control group. **D,** Endothelium-independent relaxation. There was no significant difference among all groups $(P > .05)$ in the endothelium-independent vasorelaxation induced by sodium nitroprusside. *EC,* endothelial cells.

measured eNOS mRNA expression in PCAECs by using real-time PCR analysis (Fig 3). Similarly, homocysteine alone significantly decreased eNOS expression by 59% as compared with controls $(P < .05)$. GA cotreatment dose-dependently improved eNOS mRNA levels. GA alone (100 μmol/L) did not show any effect on eNOS expression.

The expression of eNOS protein in porcine coronary arterial endothelium was depicted by using immunohistochemical staining (Fig 4). The DMSO control group showed a strong positive staining pattern of eNOS on the

endothelial layer of the arterial walls, in contrast to the poor staining pattern of eNOS in the homocysteine-alone group. It is important to note that both GA/homocysteine combined groups showed strong positive staining patterns similar to that of the control group; this indicated the recovery of eNOS protein in the endothelial layer of the GA/homocysteine-treated group.

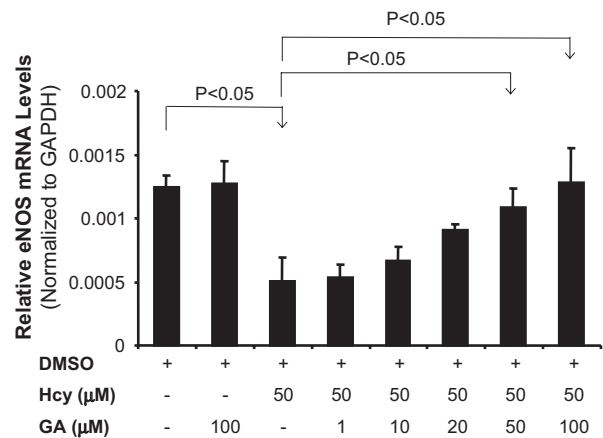**GA blocks homocysteine-induced nitric oxide decrease in PCAECs.** PCAECs were treated with homocysteine and different concentrations of GA for 24 hours. They

Fig 2. Effects of homocysteine *(Hcy)* and ginkgolide A *(GA)* on endothelial nitric oxide synthase *(eNOS)* messenger RNA *(mRNA)* levels in porcine coronary arteries. The levels of eNOS mRNA expression in porcine coronary arteries were measured by using real-time polymerase chain reaction analysis. The relative expression for eNOS in each sample was normalized against glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. The homocysteine-alone group showed a marked reduction in eNOS mRNA levels as compared with the control, and GA dose-dependently improved eNOS mRNA levels in the GA/homocysteine groups. *DMSO,* Dimethyl sulfoxide.
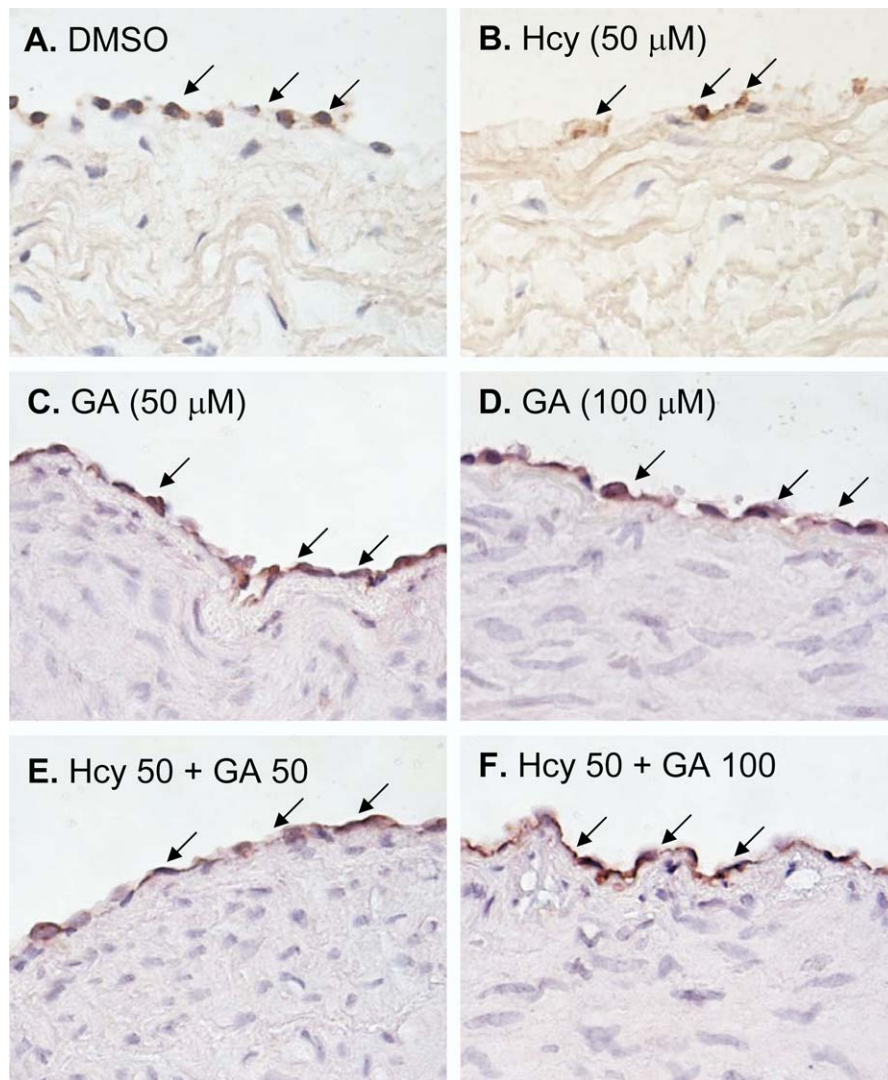

Fig 3. Effects of homocysteine and ginkgolide A *(GA)* on endothelial nitric oxide synthase *(eNOS)* messenger RNA *(mRNA)* levels in porcine coronary artery endothelial cells *(PCAECs)*. The levels of eNOS mRNA expression in PCAECs were measured by using real-time polymerase chain reaction analysis. The relative expression for eNOS in each sample was normalized against glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. The homocysteine-alone group showed a marked reduction in eNOS mRNA levels as compared with the control, and GA dose-dependently improved eNOS mRNA levels in the GA/homocysteine groups. GA alone did not show any effect on eNOS expression. *DMSO,* Dimethyl sulfoxide.

were then stained with the fluorescent dye DAF-FM DA and analyzed by using a flow cytometry assay (Fig 5). We analyzed the percentages of DAF-FM DA–staining positive cells. The homocysteine-alone group showed a 26% decrease in the nitric oxide level as compared with control, and GA at 10, 20, 50, or 100 μmol/L concentrations effectively reversed the homocysteine-induced nitric oxide decrease ($P < .05$). GA at 1 μmol/L did not reverse the homocysteine-induced nitric oxide decrease. We also measured the mean fluorescence intensity of DAF-FM DA–staining positive cells. The homocysteine-alone group showed a 38% decrease in the nitric oxide level as compared with the control, and GA at a 50 or 100 mmol/L concentration effectively reversed the homocysteine-induced nitric oxide decrease ($P < .05$). GA at 1, 10, or 20 μmol/L did not significantly reverse the homocysteine-induced nitric oxide decrease.

GA blocks homocysteine-induced superoxide anion production in porcine coronary arteries. Levels of superoxide anion produced by endothelial cells were determined by using lucigenin-enhanced chemiluminescence (Fig 6). Superoxide anion was markedly increased (by 114%) in the homocysteine-treated groups, as demonstrated by a significantly higher level of peak chemiluminescence (17.8 ± 0.54 RLU $\cdot$ s^{-1} $\cdot$ mm^{-2}; n = 8) than DMSO control (8.37 ± 1.49 RLU $\cdot$ s^{-1} $\cdot$ mm^{-2}; n = 8). Low-dose or high-dose GA alone showed no effects on the level of peak chemiluminescence as compared with control (10.9 ± 0.51 RLU $\cdot$ s^{-1} $\cdot$ mm^{-2} and 11.4 ± 1.63 RLU $\cdot$ s^{-1} $\cdot$ mm^{-2}, respectively). Again, both low-dose and high-dose GA/homocysteine combined groups showed a substantial

reduction in superoxide anion level compared with homocysteine alone, as demonstrated by a dramatically decreased level of peak chemiluminescence (12.34 ± 0.31 RLU $\cdot$ s^{-1} $\cdot$ mm^{-2} and 12.54 ± 3.51 RLU $\cdot$ s^{-1} $\cdot$ mm^{-2}, respectively).

DISCUSSION

Ginkgo biloba has a long history of clinical use with a potential benefit in cardiovascular disease, but the underlying mechanisms are not yet elucidated. This study investigated the cellular mechanism and the functional influence of GA, one of the major constituents of *Ginkgo biloba*, in protecting against homocysteine-induced endothelial damage. The study underscores that GA attenuates homocysteine-induced vasomotor dysfunction, eNOS downregulation, and free radical production. These results suggest the potential clinical application of ginkgo and its major constituents in protecting endothelium against homocysteine and superoxide-associated injuries and, thus, their potential role in cardiovascular disease prevention.

Homocysteine is a known risk factor for cardiovascular disease. Severe hyperhomocysteinemia (>100 μmol/L) has been seen in patients with homozygous deficiency of cystathionine β-synthase or methylene tetrahydrofolate reductase and is usually associated with premature arthrosclerosis or thrombosis. Even mild to moderate increases of homocysteine (10-100 μmol/L) are associated with increased risks for cardiovascular and cerebrovascular diseases. Eberhardt and colleagues[14] demonstrated that mild
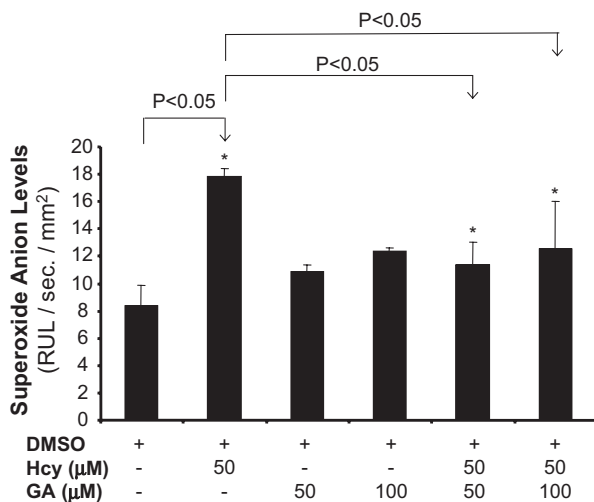
Fig 4. Effects of homocysteine *(Hcy)* and ginkgolide A *(GA)* on endothelial nitric oxide synthase (eNOS) immuno-reactivity in porcine coronary arteries. **A,** The dimethyl sulfoxide *(DMSO)* control group showed a strong positive staining pattern of eNOS on the endothelial layer of the arterial wall. **B,** The homocysteine-alone group showed a poor staining pattern of eNOS. The low-dose and high-dose GA-alone groups **(C-F)** and the low-dose and high-dose GA/homocysteine combined groups **(E-F)** showed a strong positive staining pattern similar to the control group.

hyperhomocysteinemia impaired endothelium-dependent vasodilatation by increased reactive oxygen species and a subsequent loss of nitric oxide bioavailability in an animal model. Similar findings have been validated by Lang et al[15] and Tawakol et al.[16] Our previous investigations also confirmed that homocysteine significantly decreased endothelium-dependent vasorelaxation and eNOS immunoreactivity and induced marked endothelial injury in both porcine coronary and carotid arteries.[4,6-9] Multiple studies have shown that *Ginkgo biloba* reduces vasospasm and induces endothelium-dependent relaxation. Nishida and Satoh[17] demonstrated that all constituents of *Ginkgo biloba* extract had the concentration-dependent vasorelax-ant effect on rat aortic ring strips, although the constituents had complicated interactions with each other. Similarly, Delaflotte and colleagues[18] showed the vasorelaxation effects of *Ginkgo biloba* on isolated rabbit aorta and demonstrated that relaxation was mediated in part by factors released from the endothelium. Furthermore, Auguet and Clostre[19] demonstrated that *Ginkgo biloba* increased relaxation in isolated rabbit aorta after the rapid phase of contraction and inhibited the slow phase of contraction after norepi-nephrine induction. Their findings were confirmed by Chen et al,[20,21] who demonstrated that the vasorelaxation effects of *Ginkgo biloba* on rabbit aortic rings and porcine basilar arteries were mediated via the nitric oxide pathway.
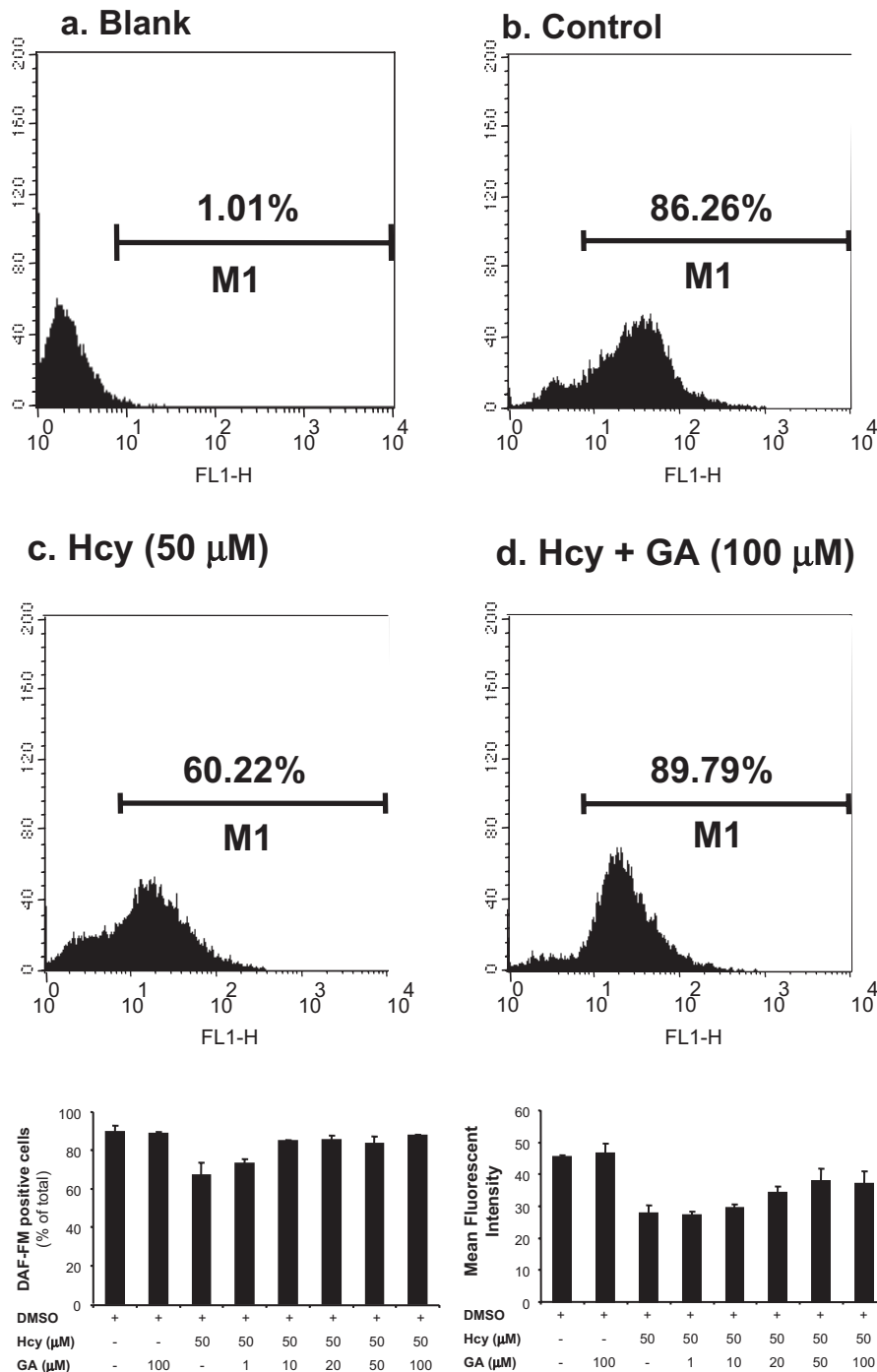
Fig 5. Effects of homocysteine *(Hcy)* and ginkgolide A *(GA)* on nitric oxide production in porcine coronary artery endothelial cells. Cells were stained with the fluorescent dye 3-Amino, 4-aminomethyl-2', 7'-difluorofluorescein Diacetate (DAF-FM DA) and analyzed by using flow cytometry assay. **A,** Representative histograms of the flow cytometry assay. **B,** Percentages of DAF-FM DA–staining positive cells. The homocysteine-alone group showed a 26% decrease in the nitric oxide level as compared with control, and GA at a 10, 20, 50, or 100 μmol/L concentration effectively reversed the homocysteine-induced nitric oxide decrease (P < .05). GA at 1 μmol/L did not reverse the homocysteine-induced nitric oxide decrease. **C,** Mean fluorescence intensity of DAF-FM DA–staining positive cells. The homocysteine-alone group showed a 38% decrease in the nitric oxide level as compared with control, and GA at 50 or 100 mmol/L effectively reversed the homocysteine-induced nitric oxide decrease (P < .05). GA at 1, 10, or 20 μmol/L did not significantly reverse the homocysteine-induced nitric oxide decrease. *DMSO,* Dimethyl sulfoxide. *FL1-H,* a fluorescence channel used in flow cytometry.

Fig 6. Effects of homocysteine *(Hcy)* and ginkgolide A *(GA)* on superoxide anion production in porcine coronary arteries. Superoxide anion was markedly increased in the homocysteine-treated groups as compared with the dimethyl sulfoxide *(DMSO)* control ($P < .05$). High-dose or low-dose GA alone showed no effects on the level of peak chemiluminescence as compared with the control. Both the high-dose and low-dose GA/homocysteine combined groups showed a substantial reduction in superoxide anion levels. *RLU,* Relative light unit.

The homocysteine concentration of 50 μmol/L chosen for this study is relevant to clinical plasma levels of mild to moderate hyperhomocysteinemia. Homocysteine at 50 μmol/L was able to induce impairment of endothelium-dependent relaxation without affecting endothelium-independent relaxation or smooth muscle contraction. The results on homocysteine-induced vasomotor dysfunction from this study are consistent with our previous studies in that homocysteine significantly impaired endothelium-dependent relaxation induced by bradykinin and exerted no significant effect on maximal contraction or endothelium-independent relaxation induced by thromboxane analogue and sodium nitroprusside, respectively.[6-9]

Our pilot study showed that the effects of GA on homocysteine-induced vasomotor dysfunction were concentration dependent. At 25 μmol/L, GA showed minimal effects on homocysteine-induced vasomotor dysfunction, whereas GA at 50 and 100 μmol/L effectively blocked the effects of homocysteine on endothelium-dependent relaxation without affecting endothelium-independent relaxation. Additionally, our study showed that GA dose-dependently attenuated the effects of homocysteine on PCAECs. To further investigate the mechanism and effects of GA and homocysteine on endothelial cells, GA concentrations of 50 and 100 μmol/L were chosen. Our study confirmed that GA by itself had no effect on the vasomotor function of porcine coronary arterial rings. However, when combined with homocysteine, both the high-dose (100 μmol/L) and low-dose (50 μmol/L) GA/homocysteine combined groups had a complete recovery of endothelium-

dependent vasorelaxation. Our vasomotor study indicates a potential clinical application of GA in preventing homocysteine-induced vascular dysfunction.

Molecular mechanisms of homocysteine-induced endothelium dysfunction have been extensively studied. Oxidative stress and eNOS reduction have been proven to be the major mechanisms through which homocysteine exerts its effects on vascular endothelial cells.[4,14,22] In this study, we investigated the molecular mechanisms of homocysteine-induced endothelial dysfunction by studying eNOS mRNA and protein production by using real-time PCR and immunohistochemical staining. To increase the quality of measurement, we used internal controls to ensure that an equal amount of tissue samples was measured for the study among all groups. The housekeeping gene GAPDH was used as the control in real-time PCR analysis. All the values were normalized before final analysis. The homocysteine-treated group showed a marked reduction of eNOS mRNA levels as compared with the control group, and a decreased eNOS protein expression in the porcine coronary arterial endothelium was depicted by immunohistochemical staining. Although this was not statistically significant, the low-dose GA/homocysteine combined group showed a partial improvement in eNOS mRNA levels, whereas the high-dose GA/homocysteine combined group showed a significant recovery of eNOS mRNA levels ($P < .05$). Furthermore, immunohistochemical staining for eNOS protein showed similar results. Endothelial NOS immunoreactivity was evaluated by gross observation of color intensity. Quantitation was not performed because of technique difficulties and the variability of staining. More reliable quantitative methods, such as Western blot for eNOS protein, may be warranted for studying the effects of homocysteine and GA on eNOS expression in coronary arteries. Nonetheless, our findings imply that GA protects the endothelium from homocysteine-induced damage with little effect on the healthy endothelium. Further studies are needed to define the underlying mechanisms of homocysteine and GA on endothelial cells.

Our study also investigated the effects of GA and homocysteine on free radical production in porcine coronary arterial rings by using lucigenin-enhanced chemiluminescence. Free radicals have been shown to play a key role in atherosclerotic plaque formation and to be involved in various vascular injuries. Several animal studies have shown the efficacy of *Ginkgo biloba* against free radical damage of the vascular endothelium. Szabo et al[23] investigated the effects of *Ginkgo biloba* in male Sprague-Dawley rats and demonstrated that *Ginkgo biloba,* like superoxide dismutase, significantly reduced the development of reperfusion-induced retinal edema and prevented neutrophil leukocyte infiltration when administered just before reperfusion. Similarly, Welt et al[24] documented the protective properties of *Ginkgo biloba* extract on endothelial cell ultrastructure, which was evident by decreased edema, luminal blebs, and pericapillary debris during acute hypoxic stress on myocardial mi-

crovessels of old rats. Additionally, Wei and colleagues[12] confirmed that *Ginkgo biloba* directly scavenged H_2O_2 in a cell-free system and decreased H_2O_2 levels in pulmonary artery endothelial cells. We further demonstrated that the homocysteine-treated group showed a significant increase in free radical production, whereas GA significantly reversed the effect of homocysteine, as indicated by dramatically decreasing levels of peak chemoluminescence in the GA/homocysteine combined groups. Our study proved that either high-dose or low-dose GA can significantly reverse homocysteine-induced free radical production.

Consistent with our previous studies,[6,7,9] GA, similar to ginsenoside Rb1, red wine, and estrogen, effectively reduced homocysteine-induced endothelial dysfunction and free radical production. These studies imply potential therapeutic strategies of using antioxidant-based pharmacotherapy to counteract the detrimental effects of hyperhomocysteinemia. Comparing the therapeutic magnitude of each agent may enhance our knowledge of these agents. However, each agent was studied under specific conditions with a concurrent local control. Many laboratory variables may influence endothelial function, and this likely invalidates the comparison. Additionally, there are no dose-dependent relationships among the several agents that we studied previously, because different concentrations were used for each agent according to its dose-dependent effects on endothelial cells. Future studies to explore the common pathway of these agents are warranted.

Admittedly, mechanisms of homocysteine-induced endothelial damage are not yet fully elucidated. Our results indicate that other cellular pathways may be involved in the functional effects of homocysteine that are not influenced by GA. As demonstrated by our data, both low- and high-dose GA significantly reversed the superoxide anion production induced by homocysteine in the homocysteine/GA combined groups, whereas low-dose GA only partially recovered the eNOS mRNA level, which did not reach statistical significance. Furthermore, the endothelium synthesizes a variety of vasodilators and vasoconstrictors. It is the ultimate balance between these and the external stimuli that determines the endothelial function. Further studies are needed to investigate the detailed molecular and cellular mechanisms of GA, as well as other endothelial-dependent factors that may play a role in endothelial dysfunction.

CONCLUSION

This study demonstrated that GA effectively blocks homocysteine-induced impairment of endothelium vasomotor function in porcine coronary arteries. In addition, GA successfully improved homocysteine-induced reduction of eNOS mRNA, protein levels, and nitric oxide production, as well as superoxide anion overproduction. This study demonstrated the functional relationship and molecular mechanisms of the effects of GA on homocysteine-induced endothelial damage. However, the observations made during this investigation do not directly translate into human arteries, and there is extremely limited study in the literature regarding serum levels of GA after oral doses in humans. Therefore, future clinical studies are warranted to determine the effects of GA on human endothelium and to determine the optimal oral dose of GA and *Ginkgo biloba*. Consistent with our previous studies, GA, similar to ginseng, red wine, and estrogen, effectively reduces homocysteine-induced endothelial dysfunction and free radical production. These studies imply an important future therapeutic strategy of using antioxidant-based pharmacotherapy to counteract the detrimental effects of hyperhomocysteinemia. Moreover, antioxidant-based pharmacotherapy, perhaps, also has a protective role in other free radical–induced vascular injuries.

AUTHOR CONTRIBUTIONS

Conception and design: WZ, HC, PHL, ABL, QY, CC
Analysis and interpretation: WZ, HC, AC, PHL, ABL, QY, CC
Writing the article: WZ, HC, ABL, QY, CC
Critical revision of the article: QY, CC
Final approval of the article: CC
Data collection: WZ, HC, AC, QY, CC
Provision of materials, patents, or resources: QY, CC
Statistical expertise: WZ, HC, CC
Obtained funding: PHL, ABL, QY, CC
Literature search: WZ, HC, CC
Administrative, technical, or logistic support: HC, QY, CC

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