EGb761 ameliorates the formation of foam cells by regulating the expression of SR-A and ABCA1: role of haem oxygenase-1

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Aims
Accumulation of foam cells in the intima is a hallmark of early-stage atherosclerotic lesions. Ginkgo biloba extract (EGb761) has been reported to exert anti-oxidative and anti-inflammatory properties in atherosclerosis, yet the significance and the molecular mechanisms of action of EGb761 in the formation of macrophage foam cells are not fully understood.

Methods and results
Treatment with EGb761 resulted in a dose-dependent decrease in oxidized low-density lipoprotein (oxLDL)-mediated cholesterol accumulation in macrophages, a consequence that was due to a decrease in cholesterol uptake and an increase in cholesterol efflux. Additionally, EGb761 significantly down-regulated the mRNA and protein expression of class A scavenger receptor (SR-A) by decreasing expression of activator protein 1 (AP-1); however, EGb761 increased the protein stability of ATP-binding cassette transporter A1 (ABCA1) by reducing calpain activity without affecting ABCA1 mRNA expression. Small interfering RNA (siRNA) targeting haem oxygenase-1 (HO-1) abolished the EGb761-induced protective effects on the expression of AP-1, SR-A, ABCA1, and calpain activity. Accordingly, EGb761-mediated suppression of lipid accumulation in foam cells was also abrogated by HO-1 siRNA. Moreover, the lesion size of atherosclerosis was smaller in EGb761-treated, apolipoprotein E-deficient mice compared with the vehicle-treated mice, and the expression of HO-1, SR-A, and ABCA1 in aortas was modulated similar to that observed in macrophages.

Conclusion
These findings suggest that EGb761 confers a protection from the formation of foam cells by a novel HO-1-dependent regulation of cholesterol homeostasis in macrophages.

Keywords
EGb761 • HO-1 • SR-A • ABCA1 • Calpain

1. Introduction
Atherosclerosis is the major death cause of coronary artery disease, which is highly associated with increased circulating cholesterol levels and inflammation in the vascular wall. Foam cells derived from macrophages play a critical role in the initiation and progression of atherosclerosis. They not only accumulate lipids but also release pro-inflammatory cytokines.1–3 Formation of foam cell is mainly due to uncontrolled uptake of modified low-density lipoprotein (LDL) or impaired cholesterol efflux in macrophages, resulting in an excessive level of lipoprotein-derived cholesterol, which consequently processed, stored, and accumulated inside the cells.1,2 Thus, therapeutic approaches by reducing foam cell formation may illuminate an efficient strategy for the prevention and treatment of atherosclerosis. The cellular cholesterol homeostasis in foam cells is dynamically regulated by cholesterol internalization and cholesterol...
efflux via scavenger receptors (SRs) and reverse cholesterol transporters (RCTs). In macrophages, class A SR (SR-A) and CD36 are responsible for internalization of modified LDL.\textsuperscript{4,5} Conversely, the efflux of accumulated cholesterol is mediated through RCTs including SR-BI, ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1).\textsuperscript{6,7} Ample evidence has demonstrated that dietary supplementation with antioxidants decreases the expression of SRs or increases expressions of RCTs, leading to the reduction of cholesterol accumulation in macrophages and atherosclerotic progression.\textsuperscript{8,9}

Ginkgo biloba extract EGb761, a widely used traditional Chinese herb for the treatment of human diseases, has been reported to display several beneficial effects in cardiovascular and nerve systems, such as the reduction of the hydroxyl radicals,\textsuperscript{10} and inflammatory response in endothelial cells.\textsuperscript{11,12} Experimentally, EGb761 has been shown to ameliorate the development of atherosclerosis in human and rodents.\textsuperscript{13,14} Despite the protective effects of EGb761 on atherosclerosis have been extensive examined, there is little information about the role of EGb761 in the setting of foam cell formation and expression of SRs and RCTs. Moreover, accumulating investigations have demonstrated that haem oxygenase-1 (HO-1), the key enzyme in the haem catabolism, participates in the anti-inflammatory or anti-oxidative effect of EGb761 in several cell types.\textsuperscript{15,16} Nevertheless, it remains unclear whether HO-1 is involved in the anti-atherogenic effect of EGb761 on the formation of foam cells.

The aim of the present study was to investigate whether EGb761 inhibits the formation of foam cells and the involving molecular mechanisms in macrophages. Our results demonstrate that EGb761 suppresses the oxLDL-mediated lipid accumulation through HO-1-dependent down-regulation of SR-A expression and up-regulation of ABCA1 expression in macrophages.

2. Methods

2.1 Reagents

EGb761 was purchased from Dr Willmar Schwabe (Germany). Rabbit anti-CD36, goat anti-SR-A, rabbit anti-3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), goat anti-LDL receptor (LDLR), rabbit anti-calpain, rabbit anti-calpastatin, rabbit anti-c-fos, rabbit anti-c-jun, rabbit anti-nuclear factor erythroid 2-related factor (Nrf2), mouse anti-Histone H1 antibodies, and Nrf2 small interfering RNA (siRNA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-sterol regulatory element binding protein 2 (SREBP2) antibody was purchased from BD Biosciences (San Jose, CA, USA). Mouse anti-ABCA1 antibody was obtained from Abcam (Cambridge, MA, USA). Rabbit anti-SR-BI and rabbit anti-ABCG1 antibodies were obtained from Novus Biologicals (Littleton, CO, USA). Mouse anti-a-tubulin antibody, phorbol myristate acetate (PMA), Ficoll/Hypaque, and human LDL were from Sigma (St Louis, MO, USA). Assay kit for calpain activity was obtained from BioVision (Lyon, France). Cholesterol assay kit was obtained from Randox (Antrim, UK). Dio-oxLDL was purchased from Biomedical Technologies (York, UK). Scrambled and HO-1 siRNA were obtained from Thermofisher Scientific (Lafayette, CO, USA). Macrophage colony stimulating factor (M-CSF) was obtained from R&D systems (Minneapolis, MN, USA).

2.2 Cell culture

Human monocyic cell line THP-1 cells (Bioresource Collection and Research Center; Hsinchu, Taiwan) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). THP-1 cells were induced with 50 nM PMA for 7 days to differentiate into macrophages. Peripheral monocytes were freshly isolated from the blood of healthy donors with the use of Ficoll/Hyphaque. The isolated mononuclear cells were incubated in RPMI 1640 medium for 1 h. Non-adherent cells were removed, and the adherent cells were grown in RPMI 1640 medium containing 10% FBS and 50 μg/mL M-CSF for 7 days to differentiate into macrophages. This protocol was approved by the Institutional Review Board at Taipei Veterans General Hospital, Taipei, Taiwan, confirming with the principles outlined in the Declaration of Helsinki (Cardiovasc Res 1997;35:2–3).

2.3 Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all animal experiments were approved by the Animal Care and Utilization Committee of National Yang-Ming University, Taiwan. Apolipoprotein E-deficient (ApoE\textsuperscript{−/−}) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were orally administrated daily with EGb761 (100 mg/kg body weight) or saline (vehicle control) by gastric gavages. After 4 weeks, mice were killed. Hearts and aortas were collected and subjected to histological examination and western blots.

2.4 Preparation of oxidized low-density lipoprotein

The oxLDL was prepared as described previously.\textsuperscript{17} LDL was exposed to 5 μM CuSO\textsubscript{4} for 24 h at 37°C and Cu\textsuperscript{2+} was then removed by extensive dialysis. The extent of modification was determined by measuring of thio-barbituric acid-reactive substances (TBARS). OxLDL containing ~30–60 mmol of TBARS defined as malondialdehyde equivalents per milligrams of LDL protein was used for experiments.

2.5 Oil red O staining

Cells were fixed with 4% paraformaldehyde and then stained by 0.5% Oil red O. Haemotoxylin was used as counterstaining. The density of lipid content was evaluated by alcohol extraction after Oil red O staining. The absorbance at 540 nm was measured using a microplate reader (BioTek Instrument, Winooski, Vermont).

2.6 Cholesterol measurement

Cellular cholesterol was extracted by hexane/isopropanol (3/2, v/v). After removing cellular debris, the supernatant was dried under nitrogen flush. The level of cholesterol was measured using cholesterol assay kits.

2.7 Cholesterol efflux assay

Cholesterol efflux experiments were performed as previously described.\textsuperscript{18} Macrophages were treated with various concentrations of EGb761 (25, 50, 100 μg/mL) for 12 h, followed by the equilibration of NBD cholesterol (1 μg/mL) for additional 6 h in the presence of EGb761. NBD cholesterol-labelled cells were washed with PBS and incubated in RPMI 1640 medium for 6 h. The fluorescence-labelled cholesterol released from the cells into the medium was measured using a multilabel counter (PerkinElmer, Waltham, MA, USA). Cholesterol efflux was expressed as a percentage of fluorescence in the medium relative to the total amount of fluorescence.

2.8 Dio-oxLDL binding assay

Dio-oxLDL uptake was performed as previously described.\textsuperscript{19} THP-1-derived macrophages were treated with or without EGb761 (25–100 μg/mL) for 24 h, followed by 10 μg/mL Dio-oxLDL at 4°C for 4 h. Cells were washed and lysates were analysed by fluorometry (Molecular Devices) using 540 nm excitation laser line and 590 nm emission filters.
2.9 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells by Tri reagent and was converted into cDNA by reverse transcriptase (Biolas, Ipswich, New England) with oligo-dT primer. The obtained cDNAs were then used as the templates for qRT-PCR. The reaction of qRT-PCR was performed by TaqMan® probe-based real-time quantification system (Foster, CA, USA). The relative amount of mRNAs was calculated with GAPDH mRNA as the invariant control.

2.10 Preparation of nuclear extracts

Cells were lysed in 10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 1 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Nuclei were pelleted at 5000 g for 5 min at 4°C. Nuclei were resuspended in lysis buffer and sheared for 15 s using microprobe sonicator and incubated on ice for 5 min. After centrifugation at 12,000 g for 5 min at 4°C, the supernatants were collected as nuclear extracts.

2.11 Immunoprecipitation assay and western blot analysis

The methods for immunoprecipitation and western blot analysis are described elsewhere. Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Aliquots (1000 μg) of cell lysates were incubated with specific primary antibody overnight, then with protein A/G-Sepharose for 2 h. Immune complexes were collected by centrifugation and then eluted in lysis buffer. Eluted protein samples were separated on SDS–PAGE. The samples were immobiloblotted with primary antibodies, then secondary antibodies conjugated with horseradish peroxidase. Bands were revealed by use of an enzyme-linked chemiluminescence detection kit (PerkimElmer, Waltham, MA, USA).

2.12 Measurement of calpain activity

Calpain activity assay was performed according to the manufacture’s protocol (BioVision). Briefly, cellular lysates (100 μg) were mixed with reaction buffer and fluorogenic substrate Ac-LLY-AFC. The level of released AFC was measured over 1 h at 37°C by fluorometry using 400 nm excitation and 505 nm emission filters.

2.13 Small interfering RNA transfection

Macrophages were transfected with scrambled, Nrf2 siRNA, or HO-1 siRNA using Lipofectamine for 24 h. After transfection, the cells were treated with EGB761 for another 24 h. The cells were then lysed for western blots and calpain activity assays.

2.14 Histological examination

Hearts were harvested from mice and fixed with 4% paraformaldehyde, embedded in paraffin, and serially sectioned at 8 μm. The deparaffinized sections were subjected to H&E staining and viewed under a Motic TYPE 102M microscope. Quantification of the atherosclerotic lesions at early-stage atherosclerosis. Modified LDL, in particular oxidized LDL (oxLDL), exerts several pro-atherogenic properties for the formation of foam cells and progression of atherosclerosis. We thus explored the effect of EGB761 on oxLDL-induced foam cell formation. Treatment with EGB761 markedly reduced the oxLDL-induced lipid accumulation in THP-1 and primary macrophages as revealed by the measurement of cellular cholesterol content or Oil red O staining (Figure 1A). To investigate the mechanisms underlying the reduction of foam cell formation by EGB761, we assessed the effect of EGB761 on cholesterol uptake and efflux. We found that EGB761 treatment significantly decreased the DiO-oxLDL-induced cholesterol uptake (Figure 1B), but significantly promoted the efficiency of cholesterol efflux (Figure 1C). Additionally, EGB761 had no effect on the protein expression of cholesterol synthesis-related genes (Figure 1D), implying de novo lipid synthesis is not involved in EGB761-mediated reduction of foam cell formation. These results suggest that the modulation of cholesterol homeostasis is a critical regulation in the suppressive effect of EGB761 during the formation of macrophage foam cells.

3. Results

3.1 EGB761 alleviates the formation of foam cells by increasing cholesterol efflux

Accumulation of lipid-laden macrophage foam cells is a hallmark of early-stage atherosclerosis. Modified LDL, in particular oxidized LDL (oxLDL), exerts several pro-atherogenic properties for the formation of foam cells and progression of atherosclerosis. We thus explored the effect of EGB761 on oxLDL-induced foam cell formation. Treatment with EGB761 markedly reduced the oxLDL-induced lipid accumulation in THP-1 and primary macrophages as revealed by the measurement of cellular cholesterol content or Oil red O staining (Figure 1A). To investigate the mechanisms underlying the reduction of foam cell formation by EGB761, we assessed the effect of EGB761 on cholesterol uptake and efflux. We found that EGB761 treatment significantly decreased the DiO-oxLDL-induced cholesterol uptake (Figure 1B), but significantly promoted the efficiency of cholesterol efflux (Figure 1C). Additionally, EGB761 had no effect on the protein expression of cholesterol synthesis-related genes (Figure 1D), implying de novo lipid synthesis is not involved in EGB761-mediated reduction of foam cell formation. These results suggest that the modulation of cholesterol homeostasis is a critical regulation in the suppressive effect of EGB761 during the formation of macrophage foam cells.

3.2 Effects of EGB761 on expression of SR-A, CD36, SR-BI, ABCA1, and ABCG1

Previous studies demonstrated that SR-A, CD36, SR-BI, ABCA1, and ABCG1 play the most critical role in cholesterol homeostasis during transformation of foam cells. In order to investigate whether the expression of these receptors and transporters is involved in the anti-atherogenic function of EGB761 in macrophage foam cells, incubation of macrophages with 100 μg/mL EGB761 time-dependently decreased the mRNA expression of SR-A without affecting the mRNA expression of CD36, SR-BI, ABCA1, or ABCG1 (Figure 2A). Moreover, EGB761 decreased the nuclear level of c-Fos and c-Jun, two key subunits of activator protein 1 (AP-1), which is crucial for gene expression of SR-A (Figure 2B). We then examined the effects of EGB761 on protein expression of SR-A, CD36, SR-BI, ABCA1, and ABCG1. As shown in Figure 3, treatment of THP-1 or primary macrophages with EGB761 (25, 50, or 100 μg/mL) for 24 h strikingly caused a decrease in the protein level of SR-A and an increase in the protein level of ABCA1 in a dose-dependent manner, whereas EGB761 did not affect protein expression of CD36, SR-BI, and ABCG1.

3.3 EGB761 increases the stability of ABCA1 protein by decreasing calpain activity

Further analysis of protein stability of ABCA1 demonstrated that, in macrophages pre-treated with CHX to block de novo protein synthesis, the degradation profile of ABCA1 protein during 12 h incubation with EGB761 was slower than the group without EGB761 treatment (Figure 4A and 4B). Additional experiments were then performed to investigate the role of calpain, a protease involved in ABCA1 proteolysis. Intriguingly, EGB761 treatment dose-dependently decreased the calpain activity (Figure 4C) without involving ABCA1 proteolysis.
affecting the expression of calpain and the endogenous inhibitor for calpain and calpastatin (Figure 4D and E). Notably, the reduced calpain activity was correlated with an increase in the protein interaction between calpain and calpastatin (Figure 4F).

3.4 HO-1 plays a key role in EGB761-mediated suppressive effects on foam cells

We additionally determined the potential role of HO-1 in EGB761-mediated protection in foam cells. Incubation with EGB761 (100 μg/mL) markedly induced protein expression of HO-1 in a dose-dependent manner in macrophages as revealed by western blots (Figure 5A). Nrf2 has been reported to play a crucial role in EGB761-mediated up-regulation of HO-1 gene expression.15 Our results showed that the nuclear level of Nrf2 protein was dose-dependently elevated upon EGB761 treatment within 60 min (Figure 5B). Moreover, transfection of the Nrf2 siRNA for gene knockdown successfully prevented the EGB761-induced increase in protein expression of HO-1 (Figure 5C). Additional experiments were then performed to investigate the involvement of HO-1 in EGB761-mediated protection in foam cells. In macrophages with HO-1 siRNA transfection at the concentration of 600 nM, the EGB761-induced HO-1 protein expression was totally abrogated (Figure 6A), whereas transfection with corresponding scrambled siRNA failed to do so. Moreover, HO-1 siRNA transfection attenuated the EGB-mediated effects on the down-regulation of c-Fos, c-Jun (Figure 6B), and SR-A protein expression (Figure 6C), up-regulation of ABCA1 protein expression (Figure 6D), inhibition of calpain activity (Figure 6E), and attenuation of lipid accumulation (Figure 6F) in macrophages, suggesting the critical role of HO-1 in the protective effects by EGB761.

3.5 EGB761 attenuates the atherogenesis in ApoE<sup>−/−</sup> mice

We additionally determined the functional significance of EGB761 in vivo. After the administration of EGB761 for 4 weeks in ApoE<sup>−/−</sup> mice, histological examination revealed that the size of atherosclerotic lesions developed in mice fed with EGB761 was significantly smaller than those found in vehicle-treated group (101.05 ± 23.33 vs. 66.21 ± 18.99 × 10<sup>3</sup> μm<sup>2</sup>) (Figure 7A). Similar to the finding of in vitro experiments, EGB761 treatment increased the protein expression of HO-1 in aortas of ApoE<sup>−/−</sup> mice (Figure 7B). We further demonstrated that EGB761 decreased the level of SR-A protein in aortas; in contrast, the level of ABCA1 protein in aortas was increased as revealed by western blots (Figure 7C).
Figure 2: Effects of EGb761 in mRNA expression of scavenger receptors and ATP-binding cassette transporters in macrophages. (A) THP-1-derived macrophages were treated with indicated concentrations (0, 25, 50, 100 μg/mL) of EGb761 for 12 h and were subjected to qRT–PCR to determine the mRNA expression of SR-A, CD36, SR-BI, ABCA1, ABCG1, or GAPDH. *P < 0.05 vs. untreated group. (B) Macrophages were treated with indicated concentrations of EGb761 for 6 h and the nuclear protein level of c-Fos, c-Jun, or Histone H1 was determined by western blotting. *P < 0.05 vs. untreated group.

Figure 3: Effects of EGb761 in protein expression of scavenger receptors and ATP-binding cassette transporters in macrophages. (A) THP-1-derived macrophages or (B) primary macrophages were treated with indicated concentrations (0, 25, 50, 100 μg/mL) of EGb761 for 24 h and the protein level of SR-A, CD36, SR-BI, ABCA1, ABCG1, or α-tubulin was determined by western blotting. *P < 0.05 vs. untreated group.
Figure 4  EGb761 increases the stability of ABCA1 protein and reduces the calpain activity. (A and B) THP-1-derived macrophages were incubated with or without EGb761 (100 μg/mL) in the presence of cyclohexamide (CHX, 2 μg/mL) for the indicated times. Cellular lysates were subjected to western blotting to determine the level of ABCA1 or α-tubulin. (C) Macrophages were treated with indicated concentrations of EGb761 for 24 h and the calpain activity was determined. (D and E) Cellular lysates were subjected to western blotting to determine the expression of calpain, calpastatin, or α-tubulin. (F) Cellular lysates were immunoprecipitated (IP) with anti-calpain or anti-calpastatin antibody and then immuno-probed (IB) with anti-calpastatin or anti-calpain antibody. *P < 0.05 vs. untreated group.

Figure 5  Nrf2 is involved in the EGb761-mediated up-regulation of HO-1. (A) THP-1-derived macrophages were incubated with indicated concentrations of EGb761 for 12 h and lysates were subjected to western blotting to determine the protein level of HO-1 and α-tubulin. (B) Macrophages were incubated with indicated concentrations of EGb761 for 60 min and nuclear extracts were subjected to western blotting to determine the level of Nrf2 and Histone H1. (C) After transfection of Nrf2 siRNA (200 nM) for 24 h, macrophages were treated with EGb761 (100 μg/mL) for additional 12 h. The expression of HO-1 or α-tubulin was examined by western blotting. *P < 0.05 vs. untreated group, #P < 0.05 vs. EGb761-treated alone group.
Figure 6  HO-1 mediates the EGb761-induced protection in macrophages. (A) THP-1-derived macrophages were transfected with various concentrations of HO-1 siRNA (150, 300, 600 nM) for 24 h, followed by EGb761 treatment (100 µg/mL) for additional 12 h. Protein expression of HO-1 and α-tubulin was measured by western blotting. (B–D) Macrophages were pre-treated with HO-1 siRNA (600 nM) for 24 h, followed by EGb761 for additional 6 h (B) or 24 h (C and D). Protein level of c-Fos, c-Jun, Histone H1, SR-A, ABCA1, and α-tubulin was determined by western blotting. (E) Calpain activity was measured by using an enzymatic method. (F) OxLDL-induced lipid accumulation was measured by alcohol extraction. *P < 0.05 vs. vehicle-treated group; #P < 0.05 vs. EGb761 alone and &P < 0.05 vs. EGb761/oxLDL-treated group.

Figure 7  EGb761 attenuates atherogenesis in ApoE−/− mice. ApoE−/− mice at 4-month age were orally administrated daily with EGb761 (100 mg/kg body weight) (n = 12) or saline (vehicle control) (n = 12) by gastric gavages for 4 weeks. (A) The atherosclerotic lesions that developed at the aortic roots were quantitated as described in Methods. (B and C) Aortas were collected from EGb761-treated ApoE−/− mice or vehicle-treated ApoE−/− mice. For each lane shown in the blotting image, aortas from three mice were pooled and lysates were subjected to western blotting to examine the level of HO-1, SR-A, CD36, SR-BI, ABCA1, ABCG1, and α-tubulin. Densitometry was the mean of the four lanes shown in the blots. *P < 0.05 vs. vehicle-treated mice.
4. Discussion

Extensive researches suggest that EGb761 is a multifunctional antioxidant providing protection from cardiovascular diseases in human and experimental rodent models.13,14 The athero-protective nature of EGb761 has been suggested. For example, EGb761 effectively inhibits the oxLDL-mediated expression of adhesion molecules and apoptosis and improves the dysfunction of endothelial nitric oxide synthase in endothelial cells.11,12 EGb761 also profoundly inhibits the proliferation of smooth muscle cells.23 Moreover, EGb761 is known to attenuate the inflammatory responses in macrophages.24 However, the efficacy of EGb761 on cholesterol metabolism in macrophage remains unclear. The results of the first part of the current study demonstrated that EGb761 alleviated the oxLDL-mediated lipid accumulation in THP-1-derived macrophages, confirming the very recent finding by Liu et al.25 that EGb761 reduces the oxLDL-induced lipid accumulation in THP-1-derived macrophages as revealed by Oil red O staining. We also found EGb761 reduced the activity of matrix metalloproteinase-9, which is a crucial regulator for plaque stability (Supplementary material online, Figure S1). We thus employed this experimental system to explore the possible molecular mechanisms underlying the EGb761-invoked inhibition of foam cell formation.

It is well established that the intracellular cholesterol homeostasis of foam cells is dynamically regulated by cholesterol uptake and efflux and these processes are tightly controlled by SRs and RCTs, respectively.4–7 We demonstrate for the first time that the suppression of foam cell formation by EGb761 is likely to be mediated through the reduction in cholesterol uptake and an increase in cholesterol efflux, but not endogenous cholesterol synthesis. Additionally, we show that EGb761 markedly decreased the mRNA and protein expression of SR-A through the inhibition of AP-1 expression without altering the expression of CD36. SR-A and CD36 are two major types of SRs involved in the uptake of oxLDL in macrophages. Studies using SR-A transgenic mice demonstrated that foam cell accumulation and atherosclerotic lesions are significantly promoted.26 Furthermore, the expression of SR-A in macrophages has been known to be regulated by antioxidants or cytokines, suggesting its crucial role in foam cell formation and the development of atherosclerosis.27–29 In view of SR-A function, the down-regulation of SR-A expression by EGb761 is likely to contribute to the decrease in oxLDL uptake and the subsequent suppression of foam cell formation.

In addition to its effect on SR-A expression, we further demonstrate that EGb761 increased the protein expression of ABCA1 without affecting its mRNA expression. Instead, this increase in ABCA1 protein expression by EGb761 appears to be a result of reduced protein degradation. ABCA1 is the major type of RCT responsible for cholesterol efflux in macrophages. The critical role of ABCA1 in maintaining cholesterol homeostasis in macrophages is well established.4,5 More importantly, our study provides the evidence for the involvement of calpain protease in this EGb761-induced post-transcriptional regulation of ABCA1, as revealed by reduced calpain activity and the increase in the interaction of calpain and its endogenous inhibitor, calpastatin, in response to EGb761 treatment. In fact, previous studies have reported that calpain plays a critical role in the stabilization of ABCA1 protein upon high-density lipoprotein or apolipoprotein Al treatment.30,31 Moreover, Wang and Oram32 demonstrated that unsaturated fatty acids phosphorylate and destabilize ABCA1 protein through a phospholipase D2/protein kinase C δ-dependent pathway. Whether this pathway is also involved in EGb761-induced decrease in calpain activity and ABCA1 protein degradation remains to be determined. However, this study identifies a unique pathway responsible for the up-regulation of ABCA1 expression induced by EGb761. Therefore, up-regulation of ABCA1 expression by EGb761 observed in this study is likely to contribute to the increase in cholesterol efflux and alleviation of foam cell formation.

The anti-oxidative activity and anti-inflammatory property of HO-1 and its metabolites, biliverdin and carbon monoxide (CO) have been claimed to provide protection in cardiovascular diseases.33 Exogenous overexpression of HO-1 by the use of agonist or adenosine-mediated gene transfer retards the progression of atherosclerosis in hyperlipidemic mice.34,35 Moreover, deletion of HO-1 promotes the gene expression of SR-A both in vitro and in vivo, leading to the exacerbation of atherosclerosis and foam cell formation.36 However, the role of HO-1 on ABCA1 expression and cholesterol efflux is largely unknown. During the past few years, considerable evidence has emerged to support the pivotal role of Nrf2/HO-1 signaling in the EGb761-mediated protection against oxidative insults.15,16 In the present study, we show that EGb761 profoundly elicited the induction of HO-1 protein in macrophages in a Nrf2-dependent mechanism. This observation provoked our interest to investigate the potential role of HO-1 in EGb761-mediated protective effect on foam cell formation. Our results showed that knockdown of HO-1 gene expression resulted in the prevention on the EGb761-induced inhibition of AP-1 expression, down-regulation of SR-A expression, up-regulation of ABCA1 expression, inhibition of calpain activity, and lipid accumulation in macrophages. Taken together, our data strongly imply that HO-1 participates in the beneficial functions of EGb761 in macrophage foam cells. On the other hand, several studies report that CO or bilirubin mediates the anti-inflammatory or anti-oxidative property of HO-1, respectively,33,34 however, whether CO or bilirubin contributes to the suppression on the formation of foam cells by EGb761 warrants further investigation.

In summary, our study provides a new insight into the crucial role of HO-1 in the EGb761-mediated anti-atherogenic property in macrophages, which reduces lipid accumulation in foam cells via a decrease in cholesterol uptake and an increase in cholesterol efflux. The cholesterol flux regulated by EGb761 is via transcriptional down-regulation of SR-A expression and post-transcriptional up-regulation of ABCA1 expression. Our findings of this study provide a novel explanation for the anti-atherogenic action by EGb761 and the possible molecular target in potential therapeutic interventions in the atherosclerosis.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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EGb761 inhibits lipid accumulation in macrophages

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