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Wiedendiol-A inhibits cholesteryl ester binding to its transfer protein

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KEY WORDS wiedendiol-A; lipoproteins; monoclonal antibodies; cholesterol esters

AIM: To study the wiedendiol-A (W-A) inhibition mechamism of plasma cholesteryl ester (CE) transfer protein (CETP) on the transfer of CE. **METHODS:** Using gel filtration method. **RESULTS:** W-A at 30 μmol · L^{-1} inhibited association of CE with CETP by 76 % and CETP transfer activity by 81 %. In addition, W-A enhanced binding of TP2, a monoclonal antibody with a CETP C-terminal epitope which is involved in CE binding, to CETP, suggesting a W-A-induced conformational change at the epitope for increased TP2 binding. When CETP activity was measured by varying high-density lipoproteins (HDL) concentration, the apparent V_{max} of CE transfer was inhibited by 74 % and 83 % in the presence of W-A at 14 and 25 μmol · L^{-1}, respectively, while the apparent K_m of HDL for CETP did not change. **CONCLUSION:** W-A action is mediated through interaction between W-A and CETP, but not through those between W-A and lipoproteins.

Elevated high-density lipoproteins (HDL) are protective for coronary disease and the activities of plasma factors to redistribute cholesteryl between lipoproteins largely determine the cholesteryl levels of HDL[1]. Plasma cholesteryl ester (CE) transfer protein (CETP) mediates the net transfer of CE from HDL to low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL). Plasma CETP is responsible for all the neutral lipid transfer activity in plasma[2] and affects the plasma HDL levels. Increased HDL in humans were observed in genetic CETP deficiency[3,4] and decreased HDL were seen in transgenic mice expressing human and monkey CETP[5,6]. Therefore, inhibiting plasma CETP may result in an increase in anti-atherogenic HDL and a decrease in atherogenic LDL and VLDL, and may be beneficial for prevention or treatment of coronary disease.

Efforts to search CETP inhibitors have yielded several compounds that displayed inhibitory activity for CETP-mediated CE transfer[7-10]. We have previously identified another small molecule, wiedendiol-A (W-A), that also inhibits the CETP transfer activity[11]. Since CETP may act by carrying CE between HDL and LDL, we tested the hypothesis in the present study that W-A inhibits CETP activity by preventing CE from binding to CETP.

MATERIALS AND METHODS

Materials CETP was obtained by stably expressing the cDNA in Chinese hamster ovary cells and purified as described previously[12]. [^{3}H]Cholesteryl oleate (2.6 GBq·mol^{-1}), horse radish peroxidase-linked goat antimouse IgG antibody, and a scintillation proximity assay (SPA) kit containing [^{3}H]CE-HDL, biotin-LDL, and streptavidin-microfluorosphere beads used for CETP activity determinations were purchased from Amersham (Arlington Heights IL). The total cholesteryl concentrations of the LDL and HDL in the SPA kit were determined to be (21.5 ± 0.6) and (2.6 ± 0.5) mg · L^{-1} ($\bar{x} \pm s$, $n = 3$) with a total-cholesteryl assay kit from Sigma. Lipids used in the experiments were purchased from Avanti Polar Lipids (Alabaster AL). The 1-Step ABTS color reagent was obtained from Pierce (Rockford IL).

Binding of cholesteryl ester by CETP The binding of CE by CETP was assessed by the procedure using phospholipid vesicles containing cholesteryl ester as a ligand donor[13]. To prepare the vesicles, egg phosphatidylcholine (PC) 5.4 μmol, cholesteryl oleate 82 nmol, and [^{3}H]cholesteryl oleate 1.5 MBq were mixed and dried in a glass tube under a stream of nitrogen. The lipids were suspended in 7 mL of buffer

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containing Tris 10 mmol · L^{-1}, edetic acid 1 mmol · L^{-1}, pH 7.4 (TE buffer) by vortexing. The solution was sonicated 4 × 12 min periods at 4 ℃ using a sonicator with a microtip (Ultrasonic W-385). The lipid solution was then centrifuged at 1700 × *g* at 4 ℃ for 5 min and chromatographed over a Sepharose 4B column (ϕ 2.6 cm × 50 cm) at a flow rate of 1 mL · min^{-1}. The central one-third of the included radioactive peak was pooled and used for experiments. The vesicles containing egg PC (7.2 nmol) with 1.5 % cholesteryl oleate (mol/mol) and [^{3}H]cholesteryl oleate 3.3 kBq of were incubated with CETP 10 μg and bovine serum albumin (BSA) 0.2 g · L^{-1} in TE buffer at 37 ℃ for 1 h in a total volume of 1 mL. The mixture was applied to a Sephadex G200 column (ϕ 18 mm × 70 mm) equilibrated with TE buffer and controlled by a fast protein liquid chromatography (FPLC) apparatus. The vesicles and CETP were separated at a flow rate of 6 mL · h^{-1} with TE buffer at 22 ℃. Fractions (3 mL) were collected and analyzed for [^{3}H]CE distribution (0.5 mL/assay) and CETP transfer activity (40 μL/assay).

Binding of mAb TP2 to CETP CETP (20 ng) was mixed with phosphate-buffered saline (PBS) 50 μL, pH 9.4 and bound to a microtiter plate overnight at 4 ℃ with slow shaking. The solution was removed and 3 % BSA (wt/vol, fatty acid free) 50 μL in PBS at pH 7.4 was added and incubated for 1 h at room temperature. After a brief wash with PBS, PBS 30 μL was mixed with 5 % Me_2SO (vol/vol) 10 μL containing appropriate concentrations of compounds and the mixture was added into the wells. The solutions were agitated at 22 ℃ for 20 min followed by addition of 10 μL of diluted mAb TP2 or a non-neutralizing mAb TP14 such that the final dilution of the mAb was 100 : 1. After a 2-h incubation, the plate was incubated with a horse radish peroxidase-linked goat antimouse IgG antibody at dilution of 200 : 1 for 2 h. The wells were then washed with PBS for 20 min and a color-forming reagent 1-Step ABTS was added for color development. The adsorbance was measured at 405 nm. Three washes with PBS 200 μL at pH 7.4 were always performed between incubations to maintain a minimal background.

CETP transfer activity kinetics HDL containing [^{3}H]CE, biotin-LDL from the SPA assay kit and CETP, were mixed in HEPES 50 mmol · L^{-1}, pH 7.4, NaCl 150 mmol · L^{-1}, 0.1 % BSA (wt/vol) to a final volume of 60 μL and W-A was added to the mixture in Me_2SO (< 1 % vol/vol). The reaction was incubated for 1 h with slow shaking and was stopped with 0.2 mL streptavidin-beads containing stop solution, incubated at 22 ℃ for 1 h and counted. Control time course experiments indicated that the assay was linear for up to 3 h under these conditions. When HDL was used as the variable in kinetic studies, linear transform was used to obtain apparent K_m and V_{max}[14]. The % of CETP inhibition was calculated by: $1 - (cpm_{compound} - cpm_{compound\ blank})/(cpm_{control} - cpm_{control\ blank}) \times 100$, where *control* and *compound* indicate the absence and presence of a compound, respectively, and *blank* indicates the adsence of CETP.

RESULTS

The inhibitory activity of W-A and several other compounds on the CE transfer activity of CETP were tested in SPA. W-A inhibited CETP CE transfer activity with an IC_{50} of 5 μmol · L^{-1} but the other hydrophobic compounds, including lovastatin, mevastatin, ACAT inhibitor CL277082, and probucol, displayed no apparent inhibition of the CETP activity at 50 μmol · L^{-1}. The results suggest that the inhibition was not caused by a general hydrophobic interaction between W-A and CETP.

The possibility that W-A blocks binding of CE to CETP was tested by a gel filtration method[13]. This method utilized phospholipid vesicles (egg PC)-containing [^{3}H]cholesteryl oleate ([^{3}H]CE) as ligand donors and incubated with CETP (as a [^{3}H]CE acceptor) followed by separation of vesicles associated- and CETP-associated [^{3}H]CE by gel filtration chromatography. The void and total inclusion volumes of the gel filtration column were approximately 50 mL and 150 mL, as determined using blue dextran and phenol red, respectively. The elution volume for CETP was approximately 80 mL. When the vesicles were chromatographed alone, [^{3}H]CE associated with the vesicles and

appeared in the void volume (Fig 1A). When CETP was incubated with the vesicles, CETP-bound [^{3}H]CE and CETP activity were both eluted at approximately 80 mL (Fig 1B). The unbound [^{3}H]CE remained in the vesicles and was eluted in the void volume (Fig 1B). Incubation of TP2, a CETP activity-neutralizing mAb, with CETP and the vesicles resulted in abolition of binding of [^{3}H]CE to CETP and CETP transfer activity at elution volume 80 mL (Fig 1C). Similarly, incubation of W-A (30 μmol·L^{-1}) with CETP and the vesicles decreased [^{3}H]CE binding to CETP transfer activity by (76 ± 5) % and (81 ± 6) % (n = 4, $\bar{x} \pm s$), respectively (Fig 1D).

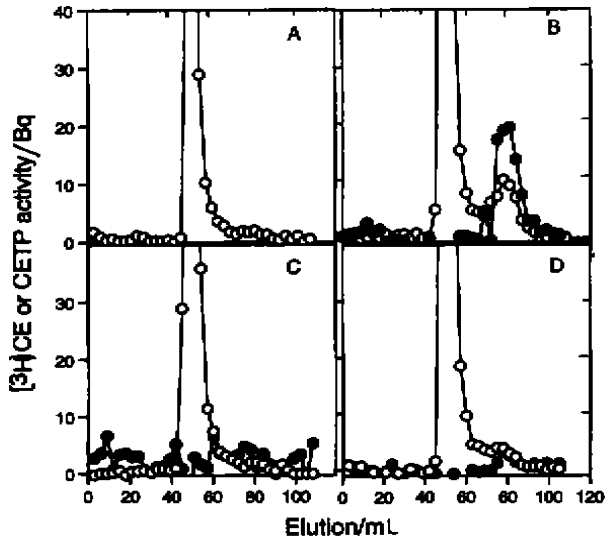

Fig 1. Effect of wiedendiol-A (W-A) on cholesteryl ester binding by CETP. (A) vesicles alone; (B) vesicles incubated with 10 μg CETP, (C) same as in (B) except TP2 200 μg was added, and (D) same as in (B) except W-A 30 μmol·L^{-1} was included in the incubation. (○) [^{3}H]CE counts and (●) CETP activity.

To test if the blockage of CE binding to CETP was a result of nonspecific hydrophobic effect from W-A, we examined the effect of another hydrophobic CETP inhibitor SCH87712[10] on [^{3}H]CE binding to CETP. Using SPA, SCH87712 inhibited CETP CE transfer activity with an IC_{50} of 20 μmol·L^{-1}. Incubation of SCH87712 with CETP and [^{3}H]CE-labeled vesicles did not decrease either CE binding to CETP or CETP CE transfer activity at elution volume 80 mL (Fig 2).

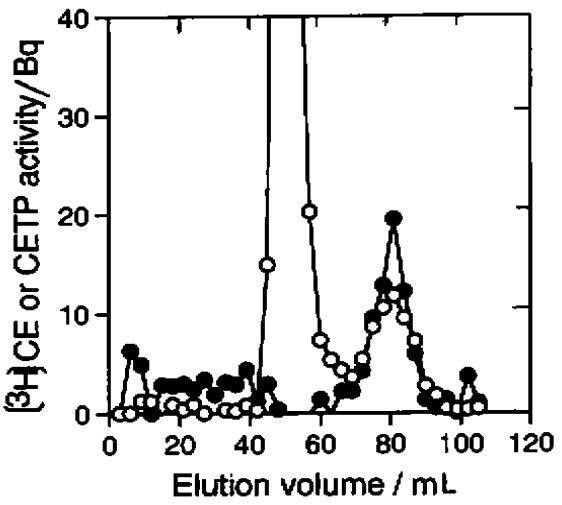

Fig 2. Effect of SCH87712 on CE binding to CETP. Incubation of SCH87712 (22 μmol·L^{-1}) with CETP and [^{3}H] CE-egg PC vesicles were performed similarly as described in Fig 1. (○) [^{3}H]CE and (●) CETP activity in column fractions, respectively.

The results suggest that SCH87712 inhibits CETP activity through a different mechanism and the inhibition of CE binding to CETP by W-A was not a nonspecific hydrophobic interaction.

Although both TP2 and W-A block CE binding to CETP, they may not bind CETP at the same site because TP2 is a large, soluble protein and W-A is a small hydrophobic molecule. To test the possibility that TP2 and W-A interact with CETP in adjacent regions involved in CE binding to CETP, effect of W-A on the binding of TP2 to CETP was examined with an ELISA method. TP2 inhibits CETP activity by binding at the C-terminus of CETP which constitues the CE binding site[13]. Increasing concentrations of W-A elevated TP2 binding to CETP by approximately 2-fold at 5 and 15 μmol·L^{-1} and by about 3-fold at 50 μmol·L^{-1} (Fig 3). In control assays, W-A did not affect the binding of mAb TP14, which did not neutralize CETP transfer activity and did not bind at the C-terminal region of CETP in the same concentration range (Fig 3).

Since SCH87712 did not block CE binding to CETP (Fig 2), SCH87712 was also examined as a control for the ability to change the binding of TP2 to CETP. SCH87712 displayed no effect on the binding of TP2, as well as TP14, to CETP up to 50 μmol·L^{-1} (Fig 3).

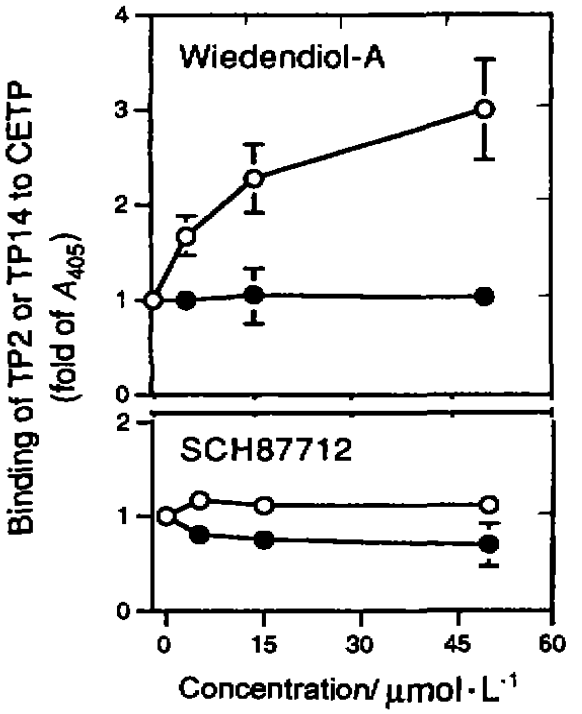

Fig 3. Interaction of TP2 with C-terminal epitope of CETP in the presence of W-A CETP was bound to a Beckman plate and then blocked by BSA. W-A or SCH87712 was incubated with CETP followed by incubation with mAb TP2 (○) or mAb TP14 (●). Absorptions of control (W-A at 0 μmol·L⁻¹) were 0.33 ± 0.03 and 0.76 ± 0.04 for TP2 and TP14, respectively. Controls for SCH87712 at 0 μmol·L⁻¹ were 0.30 ± 0.10 and 0.81 ± 0.20 for TP2 and TP14, respectively. $n = 3$, $\bar{x} \pm s$.

The nature of CETP inhibition by W-A was examined by measuring CETP activity with varying concentrations of HDL and CETP in the presence of W-A. When HDL was varied and the data were analyzed by double-reciprocal curve fitting[14], apparent V_{max} values of (33.9 ± 4.2), (9.7 ± 0.2), and (5.9 ± 0.1) TBq $[^3H]CE \cdot h^{-1} \cdot g^{-1}$ (CETP) were obtained for control (no W-A), W-A 14 and 25 μmol·L⁻¹ ($n = 3$, $\bar{x} \pm s$), respectively (Fig 4). In contrast, comparable apparent K_m values were obtained at the 3 concentrations of W-A: (1.7 ± 0.4), (1.1 ± 0.4), and (2.1 ± 0.5) mg·L⁻¹ (HDL) ($n = 3$, $\bar{x} \pm s$), respectively (Fig 4).

The data were consistent with a non-competitive inhibition by W-A, suggesting separate sites for W-A and HDL on CETP.

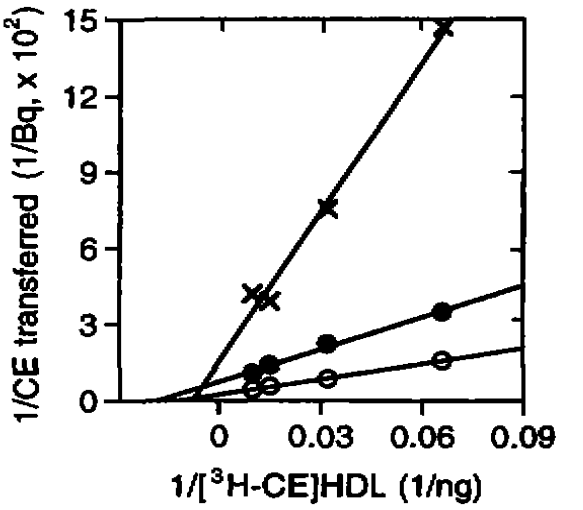

Fig 4. Kinetic analysis of effect of W-A on CETP transfer activity. Double reciprocal plot derived from measurements of CE transfer activity in the absence (○) and presence of W-A at 14 (●) and 25 μmol·L⁻¹ (×). $n = 3$, $\bar{x} \pm s$.

DISCUSSION

The present studies employed CE-CETP binding assay[13] and CETP activity kinetic measurements, and provided evidence that W-A acted through inhibiting CE binding to CETP.

The gel filtration chromatography method separated $[^3H]$CE-CETP complex from egg PC vesicles containing $[^3H]$CE. Both neutralizing mAb TP2 and W-A inhibited the binding of CE to CETP (Fig 1) whereas another hydrophobic inhibitor SCH87712 did not (Fig 2). Since CETP may transfer CE by a carrier mechanism and the binding of CE to CETP is an essential step in the transfer activity[1], blockage of CE binding to CETP by TP2 or W-A provides an efficient way to reduce CETP activity. Although both TP2 and W-A inhibit binding of CE to the transfer protein, they may not act in a common mechanism. TP2 has been shown to inhibit CETP by binding at the hydrophilic side of the C-terminal helix of CETP whereas the other side of the helix is hydrophobic and constitutes at least part of the binding site for CE[13]. The binding of TP2 may sterically inhibit CE approaching into the CE binding site. In contrast, since W-A is a small hydrophobic compound, it may bind at the opposite hydrophobic side of the helix, either directly occupying the CE binding site or alter the local conformation of the CE binding pocket to

prevent CE binding. The proximal binding sites between TP2 and W-A were supported by the ELISA experiments in which effect of W-A on enhanced binding of TP2 to CETP was observed, suggesting that the interaction of W-A changed the conformation of TP2 epitope in such a way that the affinity of TP2 for the epitope was increased (Fig 3).

The noncompetitive inhibition of CETP activity by W-A indicates that the action of W-A is not through interaction of W-A with HDL. The apparent K_m values were not significantly different (Fig 4) in the presence of W-A 0, 14, and 25 $\mu mol \cdot L^{-1}$, suggesting that the affinity of this substrate for CETP was not altered by W-A. The V_{max} was decreased in the presence of W-A 14 and 25 $\mu mol \cdot L^{-1}$ by 74 % and 83 %, respectively (Fig 4). The reduced numbers of binding sites for CE in the presence of W-A (Fig 4) is consistent with the observation that W-A prevents CE binding to CETP (Fig 1). While the CE binding site is at the C-terminus of CETP, the binding site for HDL has been shown to involve surface charged amino acid residues away from binding site of CE such as K233 and R259[15]. Therefore although W-A does not inhibit HDL binding to CETP (Fig 4), it may inhibit CETP activity by stalling the delivery of CE from the bound HDL to the CE binding pocket at the C-terminus.

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维登二醇-A 抑制胆甾醇酯及其转移蛋白的结合

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