Hibiscus Protocatechuic Acid or Esculetin Can Inhibit Oxidative LDL Induced by Either Copper Ion or Nitric Oxide Donor

Miao-Jane Lee,[†] Fen-Pi Chou,[†] Tsui-Hwa Tseng,[†] Ming-Hsun Hsieh,[†] Ming-Cheng Lin,[‡] and Chau-Jong Wang*,[†]

Institute of Biochemistry, College of Medicine, Chung Shan Medical University, No. 110, Section 1, Chien-Kuao N. Road, Taichung 402, Taiwan, and Department of Internal Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan

Oxidation of low-density lipoprotein (LDL) could increase the incidence of atherosclerosis. Previous studies have shown that copper and sodium nitroprusside (SNP) possess the ability to oxidize LDL in a dose-dependent condition. They increase the existing negative charge in LDL and increase the electrophoretic mobility. In this study, we used protocatechuic acid (PCA) and/or esculetin (ECT) to define the antioxidative activity in oxidative LDL by relative electrophoretic mobility (REM) and thiobarbituric acid-relative substances (TBARS). The data showed that ECT and PCA possessed stronger antioxidative activity than vitamin E in oxidative LDL. A previous study showed that the level of oxidative LDL can be determined by the cholesterol degradation and fragmentation of Apo B. Our results showed that Cu^{2+}-mediated oxidative LDL can induce 31% cholesterol degradation and significant fragmentation of Apo B. Both PCA and ECT exhibited remarkable ability to rescue the cholesterol degradation and Apo B fragmentation. Taken together, both PCA and ECT showed strong potency to inhibit oxidative LDL induced by copper or an NO donor. Additionally, their nontoxic characteristics elevated the possibility for their use in the daily diet; and should further prevent atherosclerosis effectively.

KEYWORDS: Protocatechuic acid; esculetin; LDL oxidation; ApoB fragmentation; cholesterol degradation

INTRODUCTION

In developed countries, atherosclerosis is one of the pathogenic causes that induces coronary heart diseases (CHD). From previous studies, we understand that atherosclerosis is closely related to oxidative LDL (*1, 2*). The oxidative LDL induced by free radical from either metal ions or nitrite enhanced the uptake of lipoprotein by macrophages, leading to cellular cholesterol accumulation and foam cell formation, the hallmark of early atherosclerosis lesions (*3−5*). Native LDL does not induce foam cell formation in cell culture; and it has been suggested that LDL must be modified (such as by oxidation, acetylation, or aggregation) before it is taken up into cells. Because oxidative LDL plays a role in causing atherosclerosis, reducing the formation of oxidative LDL becomes very important to prevention of heart diseases.

LDL contains various endogenous antioxidants including α- and γ-tocopherols, β-carotene, and retinylsterate (*6−8*). When oxidative modification of LDL occurs, these antioxidants are consumed first. Furthermore, by adding antioxidants such as α-tocopherol or dibutyl hydroxytoluene (BHT), LDL oxidation can be effectively inhibited (*9, 10*). Recently, several researchers have discovered some natural compounds possessing the anti-

Protocatechuic acid (PCA)　　　Esculetin (ECT)
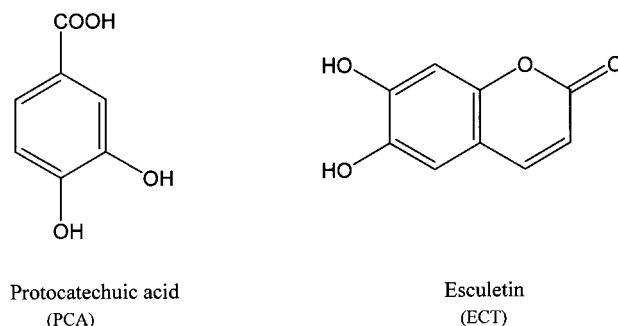
**Figure 1.** Chemical structures of PCA and ECT.

oxidative effect both to scavenge free radical and to reduce the production of oxidative LDL (*11−16*). Tomita et al. have reported that flavan-3-ols derivatives existing in tea have significant antioxidation on the Cu^{2+}-mediated oxidative modification of LDL. Several groups using different flavonoids, such as quercetin, catechin, morin, rutin, fisetin, and gossypetin, also showed that flavonoids could inhibit copper-catalyzed and macrophage-mediated LDL oxidation (*13−16*). Because most of the natural compounds extracted from foods or beverages are nontoxic, their antioxidative properties should be used widely to lower oxidative LDL.

In our previous studies, we observed that PCA and ECT (**Figure 1**) also have effective antioxidative characteristics either

* Corresponding author. Tel: +886-4-24730022 ext. 1671. Fax: +886-4-24723229. E-mail: wcj@csmu.edu.tw.
† Chung Shan Medical University.
‡ Chung Shan Medical University Hospital.
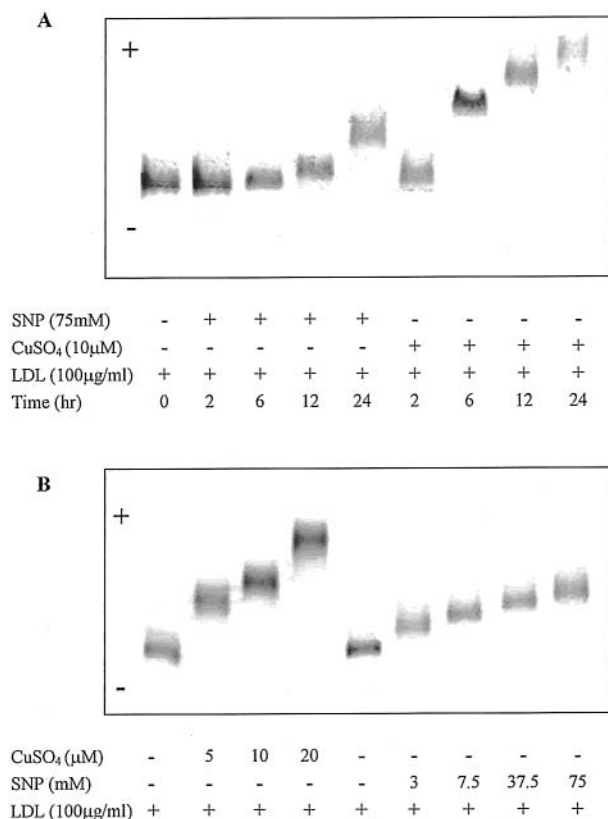
Figure 2. Oxidative modification of LDL induced by CuSO$_4$ or SNP. (A) LDL (100 μg protein/mL) was incubated with 10 μM CuSO$_4$ or 75 mM SNP at 37 °C for 0–24 h. Samples (5 μL) were separated on a Beckman paragon lipogel. After electrophoresis, the gel was stained, and the migration distance was measured. (B) LDL (100 μg protein/mL) was incubated with 0–20 μM CuSO$_4$ or 0–75 mM SNP at 37 °C for 24 h. Samples were analyzed as described in (A).

in cells or in animals (*17, 18*). PCA, a phenolic compound isolated from the dried flower of *Hibiscus sabdariffa* L. (Malvaceae) which is an ingredient of local beverages and a Chinese herbal medicine, pyrexia, is used to treat hypertension and liver damage. It has also been demonstrated recently to be an efficacious agent in inhibiting carcinogenesis because of its antioxidative feature (*19−24*). ECT is a coumarin derivative contained in many plants such as *Artemisia capillaris* (*Compositae*), the leaves of *Citrus limonia (Rutaceae)* (*25*), and *Ceratostigma willmottianum* (*26*) used as a folk medicine. It showed multiple biological activities including the inhibition of xanthine oxidase activity (*27*) and *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats (*28*), and antioxidant property (*29, 30*). Considering all these issues, we were very eager to investigate the abilities of PCA and ECT to inhibit oxidative LDL.

Because the oxidative modification in LDL gives the protein moiety of LDL an increased negative charge, oxidative LDL can be detected by electrophoretic mobility. Besides, the compositional and structural changes existing in oxidative LDL also can be observed by determining the lipid hydroperoxide contents, cholesterol degradation, and fragmentation of Apo B (*31*). In the present study, CuSO$_4$- or SNP-inducing oxidative LDL was used to investigate the antioxidation of PCA and ECT. Data showed that both PCA and ECT significantly inhibit LDL oxidation, cholesterol degradation, and ApoB fragmentation in LDL oxidized by a copper or NO donor.
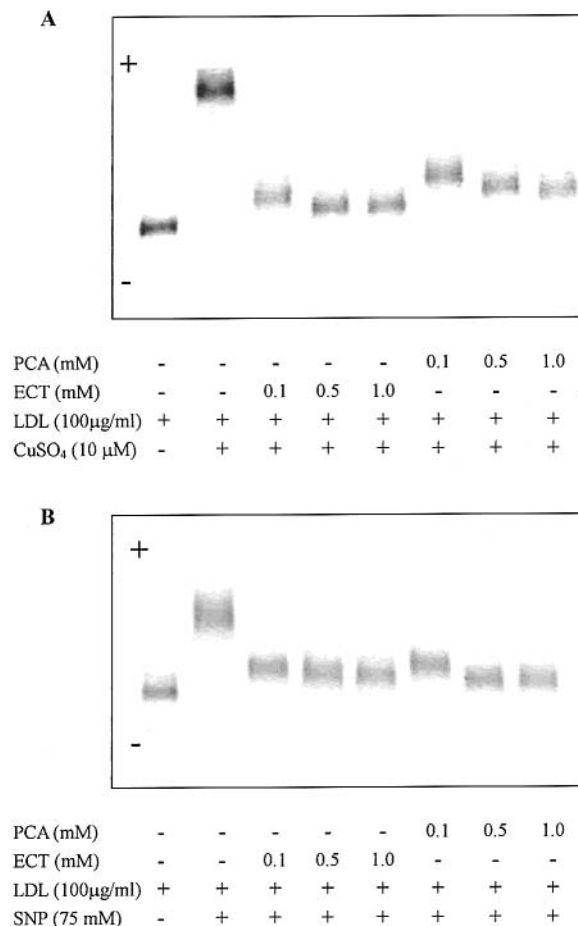
Figure 3. Inhibitory effects of PCA and ECT on the CuSO$_4$- or SNP-induced oxidative LDL. (A) LDL (100 μg protein/mL) was incubated with 10 μM CuSO$_4$ to induce oxidation. The inhibitory effects of PCA and ECT (0.1, 0.5, and 1.0 mM) on the LDL oxidation were determined by co-treatment with CuSO$_4$. The change in REM was determined. (B) 75 mM SNP was used to induce oxidative LDL, and the effects of PCA and ECT were determined as described in (A).

MATERIALS AND METHODS

Materials and Chemicals. *Hibiscus* PCA was isolated from *Hibiscus sabdariffa L.*, and its structure has been described in a previous study (*17*). ECT, curcumin (CCM), ascorbic acid (Vit. C), α-tocopherol (Vit. E), ethylenediaminetetraacetic acid (EDTA), sodium chloride, Coomassie blue, sodium nitroprusside (SNP), cupric sulfate, sodium dodecyl sulfate (SDS), polyacrylamide, thiobarbituric acid, and trichloroacetic acid were purchased from Sigma (St. Louis, MO). PD-10 column was purchased from Amersham Pharmacia Biotech (Tokyo). The Paragon lipoprotein (lipo) electrophoresis kit was purchased from Beckman Coulter (Fullerton, CA).

Isolation of LDL. Blood was obtained from healthy volunteers in the presence of 0.01% EDTA. LDL (1.019−1.063 g/mL) was isolated by sequential density ultracentrifugation at 4 °C in an Optima TL Beckman ultracentrifuge (Beckman Instruments, Mountain View, CA) as described previously (*32*). After the isolation, EDTA existing in LDL was removed by a Sephadex G-25 column (Pharmacia PD-10; bed volume, 9.1 mL) equilibration with phosphate-buffered saline (PBS). The protein content was measured using the Coomassie blue reagent (Pharmacia).

LDL Oxidation. LDL was diluted in 10 mM PBS and incubated at 37 °C in the presence of 10 μM CuSO$_4$ or 75 mM SNP. In our experiments, oxidation was carried out in the presence of PCA or ECT. After the incubation, electrophoretic mobility, lipid peroxidation, and ApoB fragmentation of the LDL were measured as described below.

Thiobarbituric Acid-Reacting Substances (TBARS). TBARS assay was performed according to the procedures of Camejo et al. (*33*).
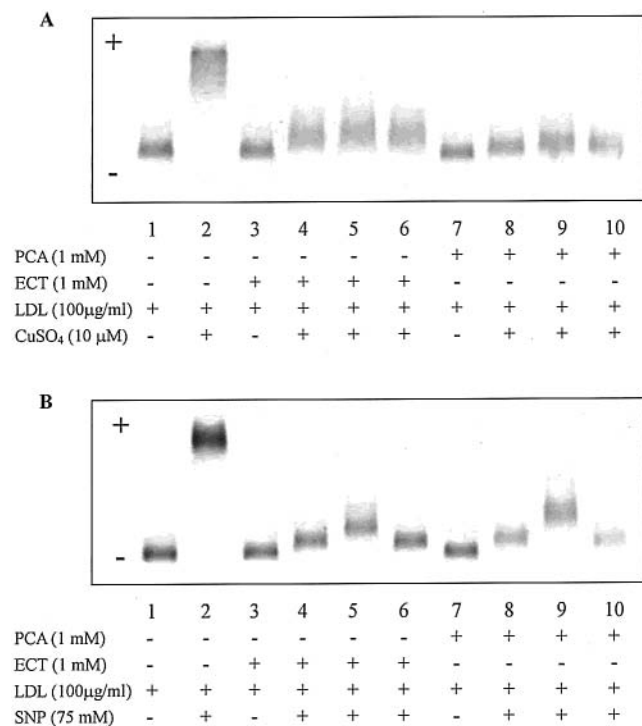
Figure 4. Inhibitory effect of PCA or ECT pre- or post-treatment on either CuSO$_4$- or SNP-induced oxidative LDL. (A) CuSO$_4$ was incubated with LDL at 37 °C for 24 h to induce oxidative LDL as a control. The samples on the gel lanes were as follows: lane 1, native LDL; lane 2, LDL and CuSO$_4$; lanes 3 and 7, LDL incubated with ECT or PCA; lanes 4 and 8, after LDL incubated with ECT or PCA for 1 h, the CuSO$_4$ was added to incubate for 24 h; lanes 5 and 9, after LDL incubated with CuSO$_4$ for 1 h, the ECT or PCA was added to incubate for 24 h; lanes 6 and 10, LDL was incubated with ECT or PCA and CuSO$_4$ synchronously for 24 h. (B) SNP was incubated with LDL at 37 °C for 24 h to induce oxidative LDL as a control. All samples on the gel lanes treated the same as in (A) except SNP was substituted for CuSO$_4$.

To each tube containing 0.55 mL of the incubated LDL, 0.5 mL of 25% (w/v) trichloroacetic acid (TCA) was added to denature protein. Then, the samples were centrifuged (10,000 rpm) at 10 °C for 30 min to remove pellet. Thiobarbituric acid (TBA; 1%, 0.5 mL) in 0.3% NaOH was added to the supernatant, and the mixed reagents reacted at 90–95 °C for 40 min in dark. After completing the reactions, samples were detected with excitation at 532 nm and emission at 600 nm in a Hitachi F2000 spectrophotofluorimeter. The concentration of TBARS is expressed as equivalents of 1,1,3,3-tetraethoxypropane that was used as standard.

Electrophoretic Mobility. The electrophoretic mobility of native or oxidized LDL was detected by agarose gel electrophoresis (*34*). The experiment was performed using Beckman paragon lipo gel electrophoresis system (Beckman Analytic, Milan, Italy). Briefly, the agarose gel (0.5% agarose and 1.0% barbital buffer) was electrophoresed (100 V) in buffer containing 10 mmol/L 5,5-diethylbarbituric acid and 50 mmol/L 5,5-diethylbarbituric acid sodium salt for 30 min. After electrophoresis, the lipoproteins in the gel were immobilized in a fixative solution (containing 60% absolute alcohol, 30% deionized water, and 10% glacial acetic acid) and the gel was dried to a film. The lipoprotein pattern was visualized by staining the film with a lipid-specific stain. This result was expressed as the distance moved from origin.

Assessment of Cholesterol Content. The amount of total cholesterol (TC) was determined enzymatically using MENAGENT Cholesterolo (Menarini Co., Italy). In the method, samples were mixed with a working solution containing cholesterol-esterase, cholesterol oxidase, and peroxidase to form chinoeimmine; and the mixture was incubated at 37 °C for 10 min. After completing the reaction, the absorbance was read at 500 nm, which is proportional to the concentration of total cholesterol.
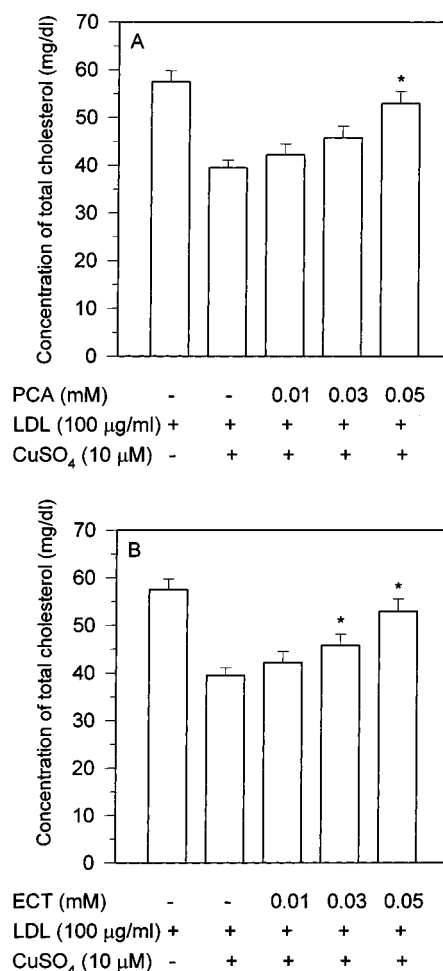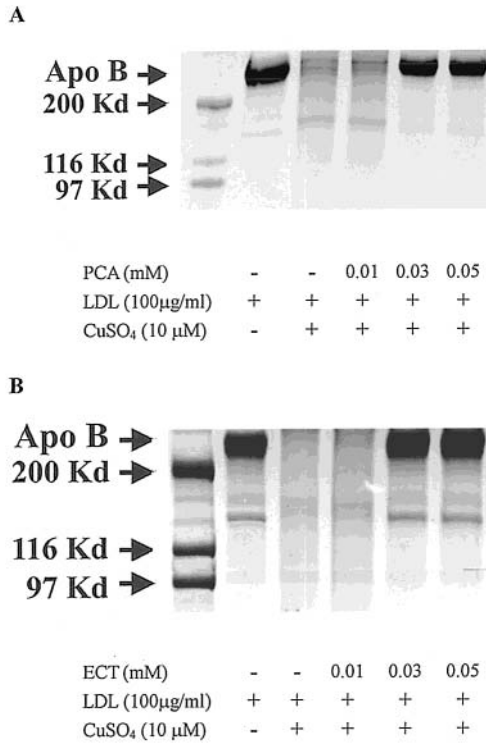

Figure 5. Inhibition of Cu^{2+}-mediated cholesterol degradation in LDL by PCA or ECT. LDL (100 μg protein/mL) was incubated with 10 μM CuSO$_4$ at 37 °C in the presence or absence of PCA (A) or ECT (B) for 12 h. After the incubation, EDTA (final concentration 1 mM) was added to prevent any further oxidation, and the total cholesterol contents were determined enzymatically. Each vertical bar represents mean ± SD for triplicate determinations. The *P* value was calculated by an unpaired *t*-test respectively compared with the control (LDL and CuSO$_4$ incubated together).

Electrophoresis of ApoB fragmentation. After the oxidation with or without antioxidants, samples were denatured with 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol at 95 °C for 5 min. SDS–polyacrylamide gel electrophoresis (SDS–PAGE, 3–15% gradient) was performed to detect the ApoB fragmentation. The electrophoresis was processed at 48 V for 150 min. After the electrophoresis, the gel was stained with Coomassie Brilliant blue R250 and dried (*11*).

RESULTS

Oxidative Modification of LDL Induced by CuSO$_4$ or SNP. The effects of various concentrations of CuSO$_4$ or SNP on inducing oxidative LDL were determined by relative electrophoretic mobility (REM) assay. The data showed that the increase in electrophoretic mobility corresponded with the oxidation time in both CuSO$_4$- and SNP-induced oxidative LDL (**Figure 2A**). When inducing for 2, 6, 12, and 24 h, the increases in CuSO$_4$-induced oxidative LDL was 1.8-, 5.8-, 7.6-, and 8.6-fold; and in SNP-induced oxidative LDL the increases were 1-, 1.4-, 2-, and 4-fold compared to that of the control (0 h). As shown in **Figure 2B**, it was observed that the higher the concentration of CuSO$_4$ or SNP, the more significant was the

PCA and ESC Inhibit LDL Oxidation

J. Agric. Food Chem., Vol. 50, No. 7, 2002   **2133**

Figure 6. Inhibition of Cu^{2+}-mediated ApoB fragmentation in LDL by PCA or ECT. LDL (100 μg protein/mL) was incubated with 10 μM $CuSO_4$ at 37 °C in the presence or absence of PCA (A) or ECT (B) for 4 h. After the incubation, EDTA (final concentration 1 mM) was added to prevent any further oxidation. 25 μg protein of LDL was applied to SDS–PAGE (3–15% gradient). After the electrophoresis, each spot was stained with Coomassie Brilliant blue R250.

oxidative level of LDL. After incubating for 24 h, the oxidative LDL induced by 5, 10, and 20 μM $CuSO_4$ were 3.6-, 4.8-, and 7.0-fold as compared to native LDL; and when induced by 0.3, 7.5, 37.5, and 75 mM SNP were 2.2-, 2.8-, 4.4-, and 5.6-fold compared to native LDL. Moreover, these data showed that $CuSO_4$ had greater ability to induce oxidative LDL than SNP.

Inhibitory Effect of PCA or ECT on either $CuSO_4$- or SNP-Induced Oxidative LDL. The 10-μM $CuSO_4$ incubated with LDL for 24 h was used to induce oxidative LDL. When the oxidation had been performed in the pretreatment of 0.1, 0.5, or 1.0 mM PCA or ECT, the significant lowering of electrophoretic mobility was as shown in **Figure 3A**. When pretreating with 0.1, 0.5, or 1.0 mM PCA, the REM was lowered by 60.21, 72.73, and 75.62%, respectively. The REM was parallel to the concentration of PCA and ECT. Treated with 0.1, 0.5, or 1.0 mM ECT prior to $CuSO_4$ and LDL, REM was reduced 81.81, 87.88, or 89.39%, respectively. On the other hand, 75 mM SNP was also used to induce oxidative LDL; and the data showed apparent changes in electrophoretic mobility in 0.1, 0.5, and 1.0 mM of PCA- or ECT-incubated. PCA at 0.1, 0.5, and 1.0 mM lowered 73.81, 88.10, and 90.48% of REM; and the same concentrations of ECT lowered 71.43, 80.95, and 80.98% of REM compared to oxidative LDL (**Figure 3B**). Additionally, as shown in **Figure 4A** and **4B**, both PCA and ECT possessed significant inhibitory effect before and after oxidative LDL inducer ($CuSO_4$ or SNP) was added.

Inhibition of $CuSO_4$-Mediated Cholesterol Degradation in LDL by PCA or ECT. Figure 5 shows the amounts of total cholesterol measured enzymatically when LDL was oxidized for 12 h in the presence of 0.01, 0.03, and 0.05 mM PCA or 0.01, 0.03, and 0.05 mM ECT. The amounts of total cholesterol
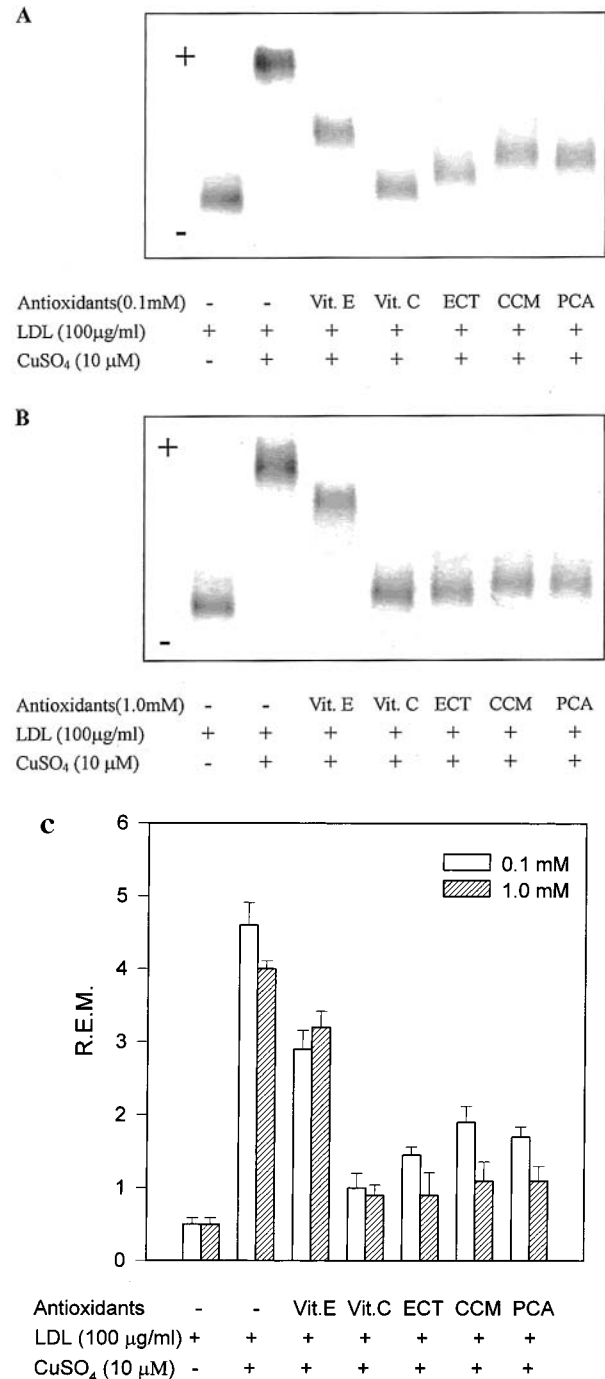
Figure 7. Comparison between PCA, ECT, and other antioxidants in inhibiting the Cu^{2+}-induced oxidative LDL. LDL (100 μg protein/mL) was incubated with 10 μM $CuSO_4$ at 37 °C in the presence or absence of 0.1 mM antioxidants (A) or 1.0 mM antioxidants (B) for 24 h. (C) The distances moved from the origin (REM) were calculated and represented as mean ± SD of triplicate determinations.

were 39.52 and 57.49 mg/dL, respectively, when LDL was incubated with or without $CuSO_4$. The decrease in total cholesterol was remarkably inhibited when LDL was oxidized in the presence of either 0.05 mM PCA or 0.07 mM ECT in 74.96% ($P < 0.01$) or 70% ($P < 0.01$).

Effects of PCA or ECT on Apo B Fragmentation. The inhibitory effects of PCA or ECT on Apo B fragmentation were studied on 3−15% SDS−PAGE (**Figure 6**). The band of Apo B was observed on native LDL which had been incubated without $CuSO_4$ for 4 h at 37 °C, but the band disappeared when

Table 1. Effect of Antioxidants on the CuSO$_4$-Induced Lipid Peroxidation in LDL

treatment[a]			TBARS (nmol/mg LDL protein)[b,c]				
antioxidant (mM)	LDL (100 μg/mL)	CuSO$_4$ (10 μM)	ECT	Vit. E	PCA	Vit. C	CCM
-	+	-	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01
-	+	+	21.62 ± 0.32	21.62 ± 0.32	21.62 ± 0.32	21.62 ± 0.32	21.62 ± 0.32
0.1	+	+	1.83 ± 0.09*	7.13 ± 0.15*	2.44 ± 0.07*	1.34 ± 0.10*	2.63 ± 0.13*
1.0	+	+	1.20 ± 0.12*	2.17 ± 0.07*	1.61 ± 0.08*	1.14 ± 0.06*	1.22 ± 0.10*

[a] LDL was pretreated with various concentrations of antioxidants at 37 °C for 1 h, and then incubated with 10 mM CuSO$_4$ for 24 h. [b] Values are the average of triplicate determinations. [c] Statistically with mean ± SD, *P < 0.0005.

Table 2. Effect of Antioxidants on the SNP-Induced Lipid Peroxidation in LDL

treatment[a]			TBARS (nmol/mg LDL protein)[b,c]				
antioxidant (mM)	LDL (100 μg/mL)	SNP (75 μM)	ECT	Vit. E	PCA	Vit. C	CCM
-	+	-	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01	0.71 ± 0.01
-	+	+	13.20 ± 0.36	13.20 ± 0.36	13.20 ± 0.36	13.20 ± 0.36	13.20 ± 0.36
0.1	+	+	1.09 ± 0.02*	2.20 ± 0.10*	1.38 ± 0.09*	1.21 ± 0.08*	1.22 ± 0.06*
1.0	+	+	1.00 ± 0.05*	9.32 ± 0.11*	1.13 ± 0.05*	0.83 ± 0.07*	1.02 ± 0.07*

[a] LDL was pretreated with various concentrations of antioxidants at 37 °C for 1 h, and then incubated with 75 mM SNP for 24 h. [b] Values are the average of triplicate determinations. [c] Statistically with mean ± SD, *P < 0.0005.

LDL was incubated with CuSO$_4$ for 4 h. In the presence of 0.05 mM PCA or ECT, the fragmentation of Apo B was inhibited and about 100% of Apo B in native LDL remained. PCA and ECT almost totally inhibited the fragmentation of apolipoprotein of LDL.
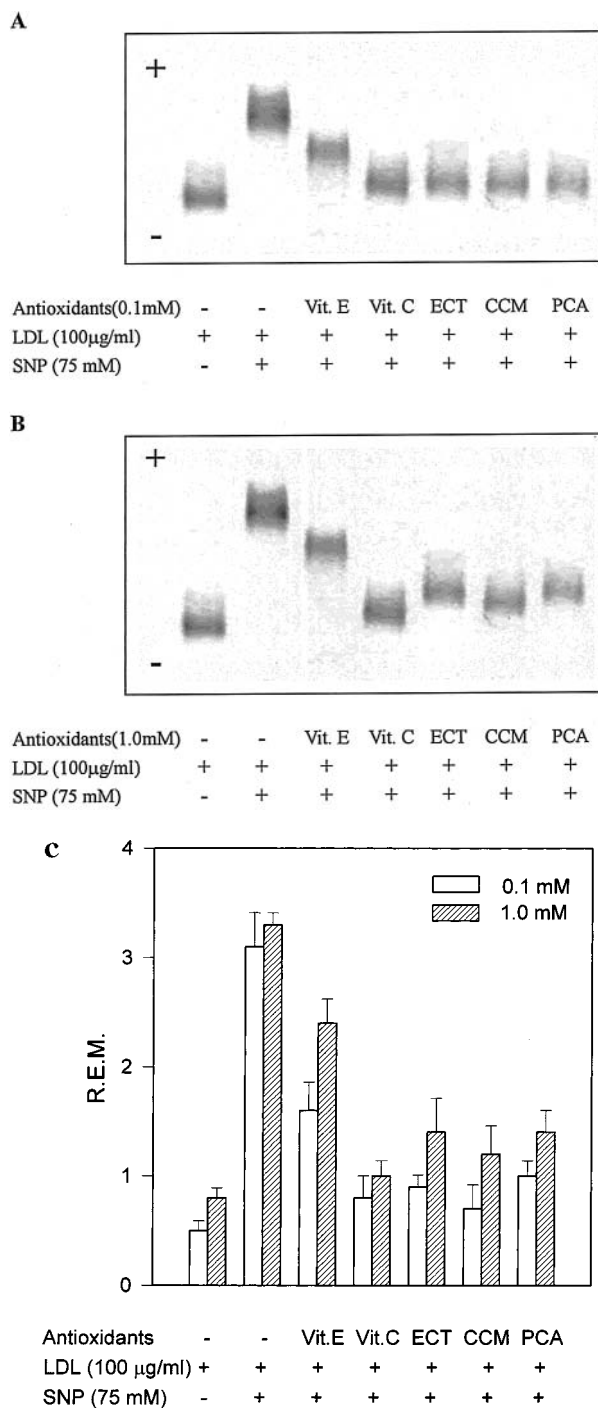
Comparison between PCA, ECT, and Other Antioxidants in Inhibiting the Oxidative LDL. We used several well-known antioxidants to compare with PCA and ECT. Compared to oxidative LDL, 0.1 mM of PCA, ECT, curcumin (CCM), Vit. C, and Vit. E reduced the REM of oxidative LDL by 70.73, 76.83, 65.85, 87.80, and 41.46%, respectively (**Figure 7A**). As shown in **Figure 7B**, 1.0 mM of PCA, ECT, curcumin (CCM), Vit. C, and Vit. E reduced the REM of oxidative LDL by 82.6, 88.57, 82.86, 88.57, and 22.86%, respectively. The data showed that the antioxidative ability of PCA and ECT was similar to that of CCM or Vit. C. In the same concentration, vitamin E showed weaker inhibitory effect than other antioxidants in adding CuSO$_4$. When oxidative LDL was induced with SNP, 0.1 mM of PCA, ECT, CCM, Vit. C, and Vit. E lowered the REM of oxidative LDL by 52, 76, 80, 4, and 60%, respectively (**Figure 8A**); and 1.0 mM of PCA, ECT, CCM, Vit. C, and Vit. E lowered the REM by 80.77, 84.62, 92.31, 88.64, and 57.69%, respectively (**Figure 8B**) compared to oxidative LDL. In low concentration, vitamin C showed weaker inhibitory effect than the other antioxidants in adding SNP; but in high concentration, vitamin E showed weaker inhibitory effect (**Figure 8C**).

Lipid Peroxidation. We also detected the lipid peroxidation induced by CuSO$_4$ or SNP. As shown in **Tables 1** and **2**, the TBARS assay showed that each antioxidant possessed inhibitory effect on lipid peroxidation as well as on lipoprotein oxidation. The results showed an increase in TBARS values when incubating the LDL with either 10 μM CuSO$_4$ or 75 mM SNP. The effect of SNP-induced oxidative LDL was weaker than that of CuSO$_4$-induced. Each antioxidant showed significant inhibition of lipid peroxidation (* P < 0.001; ** P < 0.0005). PCA and ECT, therefore, not only inhibited the fragmentation of apolipoprotein of LDL but also inhibited lipid peroxidation. In addition, the antioxidative effect of PCA or ECT was similar to that of other well-known antioxidants.

DISCUSSION

Oxidized LDL could promote atherogenesis by its cytotoxicity, its chemotactic effects on monocytes, its inhibitory effects on macrophage motility, and its uptake by the macrophage scavenger receptor, resulting in stimulation of cholesterol accumulation and hence foam cell formation (*35*). It would therefore be important to inhibit the oxidative modification of LDL to prevent atherosclerosis. Here it was observed that PCA and ECT have efficacious antioxidative characteristics to reduce oxidative LDL. PCA is an ingredient of local beverages and ECT is a coumarin derivative used as folk medicine. Both of them possess the advantage of being nontoxic and can be used in the daily diet. Some antioxidants that inhibit LDL oxidation in vitro, for instance BHT (*10*), suppress experimental atherosclerosis in animals when administered in the diet. Since PCA and ECT exhibited strong antioxidative effects in our results, the ability of PCA or ECT to inhibit LDL oxidation in cells or in animals remained to be further investigated.

In the present study, several antioxidants were used to compare their antioxidative ability with PCA or ECT. The results showed that PCA, ECT, curcumin, and vitamin C possessed similar abilities to inhibit the Cu^{2+}- or SNP-induced oxidative LDL. Vitamin E showed a weaker ability to inhibit oxidative LDL. The data also demonstrated that treatment with PCA or ECT inhibited either SNP- or Cu^{2+}-mediated LDL oxidation in a concentration-dependent manner. The mechanisms are still uncertain, and require further studies. According to some, flavonoids are known to bind copper ions (*11*); it is possible that PCA and ECT may exert part of their effect by reducing the formation of free radicals mediated by copper ions in the cell-free system. On the other hand, because Cu^{2+}-mediated cholesterol degradation in LDL was significantly inhibited in the presence of PCA or ECT, the effect of PCA or ECT may be to protect LDL from forming 7-hydroperoxy or hydroxy sterol (*11, 36, 37*). In a previous study, oxidative LDL was reported to generate peroxy radicals leading to a chain reaction and resulting in an amplified number of lipidperoxides. The results can be observed by TBARS. The large amounts of lipid peroxides accompanied by rearrangement of the fatty acid double bond yielded conjugated diene to induce cholesterol degradation. Consequent to the propagation reactions, fatty acid

PCA and ESC Inhibit LDL Oxidation

J. Agric. Food Chem., Vol. 50, No. 7, 2002 **2135**

Figure 8. Comparison between PCA, ECT, and other antioxidants in inhibiting the SNP-induced oxidative LDL. LDL (100 μg protein/mL) was incubated with 75 mM SNP at 37 °C in the presence or absence of 0.1 mM antioxidants (A) or 1.0 mM antioxidants (B) for 24 h. (C) The distances moved from the origin (REM) were calculated and represented as mean $\pm$ SD of triplicate determinations.

fragmentation occurs, leading to the formation of highly reactive intermediates, such as aldehydes and ketones, which can then complex with the adjacent apo B to induce apo B fragmentation (*38*). The present data showed that PCA or ECT inhibited all changes occurring in oxidative LDL, including the lipid peroxidation, cholesterol degradation, apo B fragmentation, and ion increase of protein moiety. These mechanisms could be involved in exploring the real inhibitory pathway of PCA or ECT.

Whether or not dietary PCA or ECT have any effect on LDL oxidation in vivo, and therefore influence the atherosclerotic process, remains to be established. The above findings present the possibility that PCA or ECT could protect the oxidative modification of LDL in atherosclerotic lesion, and may be natural anti-atherosclerotic components of the diet, though this assumption requires further experiments, especially regarding their pharmacological properties and mechanisms in macrophages or in animals.

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