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Cacao Polyphenols Influence the Regulation of Apolipoprotein in HepG2 and Caco2 Cells

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ABSTRACT: Cocoa powder is rich in polyphenols, such as catechins and procyanidins, and has been shown to inhibit low-density lipoprotein (LDL) oxidation and atherogenesis in a variety of models. Human studies have also shown daily intake of cocoa increases plasma high-density lipoprotein (HDL) and decreases LDL levels. However, the mechanisms responsible for these effects of cocoa on cholesterol metabolism have yet to be fully elucidated. The present study investigated the effects of cacao polyphenols on the production of apolipoproteins A1 and B in human hepatoma HepG2 and intestinal Caco2 cell lines. The cultured HepG2 cells or Caco2 cells were incubated for 24 h in the presence of cacao polyphenols such as (−)-epicatechin, (+)-catechin, procyanidin B2, procyanidin C1, and cinnamtannin A2. The concentration of apolipoproteins in the cell culture media was quantified using an enzyme-linked immunoassay, and the mRNA expression was quantified by RT-PCR. Cacao polyphenols increased apolipoprotein A1 protein levels and mRNA expression, even though apolipoprotein B protein and the mRNA expression were slightly decreased in both HepG2 cells and Caco2 cells. In addition, cacao polyphenols increased sterol regulatory element binding proteins (SREBPs) and activated LDL receptors in HepG2 cells. These results suggest that cacao polyphenols may increase the production of mature form SREBPs and LDL receptor activity, thereby increasing ApoA1 and decreasing ApoB levels. These results elucidate a novel mechanism by which HDL cholesterol levels become elevated with daily cocoa intake.

KEYWORDS: Cacao polyphenols, procyanidins, apolipoprotein A1, apolipoprotein B, HepG2, Caco2, atherosclerosis

INTRODUCTION

Hypercholesterolemia is a major risk factor for atherosclerosis, especially when there is a significant increase in low-density lipoprotein (LDL) cholesterol levels.[1] However, prospective studies have shown there is a negative correlation between plasma high-density lipoprotein (HDL) cholesterol and cardiovascular disease.[2] Such studies suggest that careful monitoring of both LDL and HDL cholesterol levels is important for preventing atherosclerotic lesions.

Epidemiological studies have demonstrated a negative correlation between mortality from coronary/ischemic heart disease and consumption of plant polyphenols.[3,4] In the Zutphen Elderly Study, intake of catechins was shown to associate inversely with ischemic heart disease, but had no correlation with the incidence of myocardial infarction.[3]

Apolipoprotein A1 (ApoA1) is the major protein component of HDL particles in plasma. These antiatherogenic particles transport cholesterol from extrahepatic tissues to the liver for further processing and excretion in bile acid. Apolipoprotein B (ApoB) is synthesized primarily by intestinal and liver cells and then acts as the core protein of LDL and very low density lipoprotein particles.

Several types of polyphenolic compounds, such as isoflavones, have been reported to increase ApoA1 levels in the hepatic cell line, HepG2.[5,6] Furthermore, citrus flavonoids and soy isoflavones have been shown to decrease ApoB levels in HepG2 cells,[7−10] whereas red wine polyphenols were found to decrease ApoB levels in both HepG2 cells and the intestinal cell line Caco2.[11,12]

Cacao liquor, which is produced by fermentation and roasting of cacao beans, is one of the main ingredients of cocoa and chocolate and is rich in polyphenols with antioxidative activity.[13] Epicatechin, catechin, and procyanidins (catechin oligomers) are the main polyphenolic components of cacao liquor. There is evidence that dark chocolate dietary supplementation increases plasma HDL cholesterol and inhibits oxidation of LDL cholesterol in healthy human volunteers.[14] In addition, we have shown that daily consumption of cocoa powder decreases plasma LDL cholesterol and increases HDL cholesterol levels in healthy human subjects.[15]

In the present study, we aimed to elucidate how particular cacao polyphenols affect cholesterol metabolism. To achieve this objective, we conducted an in vitro study in which HepG2 or Caco2 cells were incubated with cacao polyphenols and then changes in ApoA1 and ApoB protein levels and mRNA expression were determined. In addition, we examined the effect of cacao polyphenols on LDL receptors and sterol regulatory element binding proteins (SREBPs) in HepG2 cells.

MATERIALS AND METHODS

Materials. Human transformed hepatic HepG2 and intestinal Caco2 cells were purchased from Riken Cell Bank (Ibaraki, Japan). Cell culture medium was purchased from Sigma (St. Louis, MO), and 10 cm cell culture dishes and 6-well (35 mm) culture plates were from Falcon (BD Biosciences, San Diego, CA). Cacao polyphenols such as procyanidin B2 (B2), procyanidin C1 (C1), cinnamtannin A2 (A2), and cacao liquor procyanidins (CLPr) were prepared from cacao liquor

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according to the method described in our previous paper.[16] CLPr was made from cacao liquor as follows. Briefly, cacao liquor was defatted with *n*-hexane and the residue extracted with acetone. The *n*-butanol soluble fraction of the extract was applied to a Diaion HP2MG column (Mitsubishi Kasei Co. Ltd., Tokyo, Japan), and the fraction eluted with 80% ethanol was collected and freeze-dried for use in the experiments. The composition of CLPr was as follows: total polyphenols (measured by Prussian blue method as epicatechin equivalents), 79.3%; (+)-catechin, 2.5%; (−)-epicatechin, 5.9%; procyanidin B2, 4.0%; procyanidin C1, 2.6%; and cinnamtannin A2, 3.2%. Procyanidin B2, procyanidin C1, and cinnamtannin A2 were prepared from CLPr using preparative HPLC. (−)-Epicatechin (EC) was obtained from Sigma and (+)-catechin from Extrasynthese (Genay, France).

Cell Cultures. The HepG2 or Caco2 cells were grown to near confluence in monolayer cultures at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). The cells were then washed in PBS and incubated for 24 h in 6-well culture plates containing DMEM without FBS and 0.1−10 μM concentrations of EC, CA, B2, C1, A2, or CLPr in ethanol. CLPr was calculated as epicatechin equivalents. The final concentration of ethanol added to the cell cultures was <0.05% (v/v).

Quantification of Apolipoprotein Levels in Culture Media. The concentrations of ApoA1 and ApoB in the cell culture media were quantified using an enzyme-linked immunoassay (ALerCHEK, Portland, ME), according to the manufacturer's instructions. Protein concentrations were determined in 96-well plates using a protein assay kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions.

Quantitative Real Time RT-PCR Assay of mRNA Expression. Total RNA was extracted with TRI-zol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The extract was then purified using the RNeasy mini kit (Qiagen, Germantown, MD) and the total RNA reverse-transcribed using the RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). The resulting cDNA was used for real time quantitative PCR in an ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA). All of the specific primers and TaqMan probes (assays on demand gene expression assay) were obtained from Applied Biosystems. The results for each sample were standardized to expression of GAPDH mRNA.

LDL Receptor Binding Activity. HepG2 cells were grown in 12-well culture plates, followed by washing in PBS and incubation for 24 h in DMEM serum-free media containing 10 μM EC, CA, B2, C1, A2, or CLPr in ethanol. The cells were then washed in PBS and incubated for a further 6 h in DMEM serum-free media containing fluorescent-labeled Dil-LDL (10 mg/L, Biomedical Technologies, Stoughton, MA). The cells were washed twice with DMEM and treated with trypsin to produce a suspension of single cells. The fluorescence of the cell suspensions was measured at 514 nm (excitation) and 550 nm (emission) wavelengths.

Measurement of Sterol Regulated Binding Protein. Following incubation, the cells were washed in PBS and then treated with lysis buffer. Protein concentrations were determined in 96-well plates using a protein assay kit (Bio-Rad), according to the manufacturer's instructions. The cell protein extracts were loaded (30 μg of protein/lane) onto 2−15% gradient polyacrylamide gels, electorophoresed, and then transferred onto PVDF membranes (Amersham Biosciences, NSW, Australia). The membranes were blocked with immunoblock (DS-Pharma, Osaka, Japan) and incubated overnight at 4 °C in Tris-buffered saline containing 0.1% Tween-20 and either anti-SREBP-1 (sc-367) or SREBP-2 (sc-5603) (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with horseradish peroxidase conjugated secondary antibody for 24 h at room temperature and the antibody complexes visualized using ECL+ detection reagent (Amersham Biosciences), according to the manufacturer's instructions. Chemiluminescence signals were detected using Cemi-Doc XRS (Bio-Rad) and the relative signals determined using β-actin as a reference.

Statistical Analysis. The data are expressed as means ± SD. The analyses were carried out using SPSS statistical software (SPSS Inc., Chicago, IL). When ANOVA revealed $p < 0.05$, the data were further analyzed using Scheffe's multiple-range test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

ApoA1 Production and Secretion in HepG2 and Caco2 Cells. In the HepG2 study, all compounds increased ApoA1 expression in a dose-dependent manner (Table 1). Expression of ApoA1 protein increased significantly in the presence of EC, CA, C1, and CLPr at a concentration of 10 μM. This was especially apparent with CLPr, with significant differences from the control being observed over the concentration range of 0.1−10 μM. All samples of the HepG2 cultures containing 10 μM levels of the polyphenols showed a significant increase in ApoA1 mRNA expression. There was a positive correlation between ApoA1 mRNA expression and protein production, although this relationship was not statistically significant. Both B2 and A2 at a concentration of 10 μM caused a significant up-regulation in ApoA1 mRNA expression, although no changes in protein production were observed in these cultures.

In the Caco2 cell study, a similar tendency of increased ApoA1 production was also observed with the various polyphenols (Table 1). However, polyphenol-triggered up-regulation of ApoA1 in Caco2 cells appeared to be slightly weaker than in HepG2 cells, with the exception of A2. Significant differences in ApoA1 production were observed for A2 over the concentration range of 1.0−10 μM. In contrast, significant increases in ApoA1 production occurred only at the highest concentration of EC and CLPr (10 μM).

In the Caco2 cells, ApoA1 mRNA expression was unchanged for most polyphenols with the exception of A2-treated cells, for which an increase was observed. A similar result for protein production was observed, with A2 at concentrations of 1.0 and 10 μM causing a significant increase in ApoA1 protein expression.

ApoB Production and Secretion in HepG2 and Caco2 Cells. Polyphenol treatment resulted in a dose-dependent decrease in ApoB protein levels in both HepG2 and Caco2 cells, with the exception of CA (Table 2). In the HepG2 cells, EC and A2 at a concentration of 10 μM and B2 at a concentration of 0.1 or 10 μM caused a significant decrease in ApoB protein production. In the Caco2 study, treatment with C1 at 1.0 and 10 μM concentrations and B2 and A2 at 10 μM concentrations also caused a significant decrease in ApoB protein levels.

In the HepG2 cells, all of the polyphenols decreased ApoB mRNA expression in a dose-dependent manner. A significant decrease in ApoB mRNA expression was caused by 10 μM A2 and CLPr, 1.0 and 10 μM EC and B2, and 0.1 μM CA.

In the Caco2 samples, treatment with different concentrations of polyphenols decreased ApoB mRNA expression, with the exception of C1 and A2 at concentrations of 1.0 and 10 μM, which tended to increase ApoB mRNA expression. No such changes were observed in protein secretion.

Expression of mRNA in HepG2 Cells. We examined mRNA expression in HepG2 cells following treatment with different concentrations of the polyphenolic compounds (Table 3). The expression of LDL receptor (LDLR) mRNA increased in the presence of polyphenols compared with control cultures. In the presence of CLPr this change occurred in a dose-dependent

Table 1. Effect of Each Cacao Polyphenol on ApoA1 Protein Levels and mRNA Expression in HepG2 and Caco2 Cells[a]

	ApoA1 protein (μg/mg of protein)		ApoAl mRNA (relative expression)	
treatment (μM)	HepG2	Caco2	HepG2	Caco2
EC				
0	361 ± 22.4	116 ± 3.0	1.00 ± 0.00	1.00 ± 0.00
0.1	390 ± 15.8	147 ± 11.1	1.23 ± 0.17	0.98 ± 0.31
1.0	417 ± 18.5	157 ± 12.9	1.53 ± 0.16**	1.17 ± 0.28
10	467 ± 30.5*	161 ± 13.1*	1.95 ± 0.29**	0.88 ± 0.25
CA				
0	305 ± 14.3	189 ± 20.1	1.00 ± 0.00	1.00 ± 0.00
0.1	329 ± 9.8	167 ± 11.9	1.09 ± 0.14	1.28 ± 0.15
1.0	346 ± 11.7	225 ± 31.6	1.52 ± 0.15**	1.12 ± 0.20
10	400 ± 17.6**	216 ± 18.6	2.12 ± 0.39*	1.03 ± 0.07
B2				
0	379 ± 19.5	112 ± 2.8	1.00 ± 0.00	1.00 ± 0.00
0.1	387 ± 18.3	113 ± 3.8	1.19 ± 0.12	0.92 ± 0.20
1.0	403 ± 28.9	111 ± 4.6	1.26 ± 0.12	0.87 ± 0.10
10	444 ± 27.7	130 ± 5.8	1.44 ± 0.15*	0.70 ± 0.04
C1				
0	329 ± 8.4	159 ± 5.4	1.00 ± 0.00	1.00 ± 0.00
0.1	339 ± 10.7	182 ± 12.1	1.19 ± 0.10	1.77 ± 0.56
1.0	410 ± 46.8	172 ± 2.9	1.53 ± 0.24	1.30 ± 0.38
10	480 ± 32.5**	198 ± 10.5	2.16 ± 0.28**	1.93 ± 0.58
A2				
0	321 ± 28.5	149 ± 32.5	1.00 ± 0.00	1.00 ± 0.00
0.1	341 ± 17.2	142 ± 18.1	1.22 ± 0.14	0.98 ± 0.09
1.0	356 ± 15.1	244 ± 32.6**	1.55 ± 0.28	2.59 ± 0.39**
10	372 ± 20.2	272 ± 29 3**	1.51 ± 0.20*	2.89 ± 0.65*
CLPr				
0	291 ± 11.9	150 ± 9.8	1.00 ± 0.00	1.00 ± 0.00
0.1	335 ± 13.8*	161 ± 16.7	1.40 ± 0.16*	1.33 ± 0.22
1.0	359 ± 23.2*	173 ± 9.3	1.60 ± 0.12**	0.99 ± 0.19
10	360 ± 16.7**	173 ± 7.5*	2.43 ± 0.35**	1.16 ± 0.20

[a] The data are expressed as means ± SEM (n = 6). Asterisks (*) denote a significant difference compared to control cultures (concentration 0): *, $p < 0.05$; **, $p < 0.01$.

manner and at a concentration of 10 μM was significantly different from controls. Treatment with CA or CLPr resulted in a significant increase in mRNA expression for scavenger receptor BI (SR-BI), ATP binding cassette transporter A1 (ABCA1), sterol regulatory element binding family 1 (SREBF1), and SREBP cleavage activating protein (SCAP) compared with control cells. SR-BI expression was up-regulated following the addition of CA and CLPr in a dose-dependent manner, although this change did not occur with the other compounds. The mRNA expression for ABCA1 and SREBF1 increased in the presence of the polyphenols in a dose-dependent manner, whereas expression of SCAP mRNA increased for CA, C1, A2, and CLPr, but not for EC or B2.

LDL Receptor Binding Assay. LDL receptor binding activity was determined by calculating the mean value of two independent experiments. LDL receptor binding activity in HepG2 cells was enhanced by treatment with cacao polyphenols (Figure1). In particular, 10 μM CLPr increased this activity 1.61-fold compared with controls. Comparison of the results from different compounds showed LDL receptor binding activity in HepG2 cells correlated with the degree of polymerization of the procyanidins.

Expression of SREBPs. The level of SREBP expression was calculated as the mean of two independent experiments. Pretreatment with 10 μM levels of the cacao polyphenols resulted in a marked increase in mature SREBP1 and SREBP2 levels compared with controls (Figure 2). The increase in SREBP1 up-regulation relative to control values caused by a 10 μM concentration of the different polyphenols occurred in the following order: EC (6.56-fold) > CLPr (5.77-fold) > B2 (5.09-fold) > A2 (4.91-fold) > C1 (2.66-fold) > CA (2.41-fold). Comparison of the results from different compounds showed SREBP1 expression in HepG2 cells decreased with the degree of polymerization of procyanidin. However, the expression with the exception of CA was the lowest of the polyphenol samples. B2, C1, and A2 are polymers of EC. The configuration of the hydroxyl group on the C ring is different between CA and EC. The increase in SREBP2 expression relative to control values caused by a 10 μM concentration was C1 (27.42-fold) > B2 (20.19-fold) > A2 (13.81-fold) > EC (8.61-fold) > CA (7.81-fold) > CLPr (2.61-fold). SREBP2 expression in HepG2 cells increased with the degree of polymerization of the procyanidin to the trimer.

Table 2. Effects of Each Cacao Polyphenol on ApoB Protein Levels and mRNA Expression in HepG2 and Caco2 Cells[a]

	ApoB protein (μg/mg of protein)		ApoB mRNA (relative expression)	
treatment (μM)	HepG2	Caco2	HepG2	Caco2
EC				
0	344 ± 19.7	108 ± 8.91	1.00 ± 0.00	1.00 ± 0.00
0.1	307 ± 14.6	93 ± 6.62	0.90 ± 0.12	0.94 ± 0.28
1.0	298 ± 19.0	87 ± 3.90	0.65 ± 0.04**	1.04 ± 0.35
10	286 ± 14.0*	62 ± 3.43	0.66 ± 0.07**	0.81 ± 0.34
CA				
0	384 ± 49.5	115 ± 11.7	1.00 ± 0.00	1.00 ± 0.00
0.1	334 ± 18.1	92 ± 6.10	0.78 ± 0.08*	1.18 ± 0.11
1.0	307 ± 16.6	113 ± 9.75	0.89 ± 0.15	1.00 ± 0.13
10	307 ± 23.4	104 ± 6.96	0.82 ± 0.10	0.96 ± 0.05
B2				
0	490 ± 34.6	99 ± 14.6	1.00 ± 0.00	1.00 ± 0.00
0.1	380 ± 16.2*	87 ± 15.31	0.84 ± 0.08	0.99 ± 0.22
1.0	376 ± 17.2	59 ± 10.37	0.71 ± 0.06**	0.95 ± 0.18
10	332 ± 13.7*	50 ± 5.48*	0.55 ± 0.07**	0.84 ± 0.14
C1				
0	454 ± 35.9	102 ± 4.86	1.00 ± 0.00	1.00 ± 0.00
0.1	383 ± 22.5	90 ± 5.97	0.88 ± 0.17	1.85 ± 0.66
1.0	354 ± 15.3	80 ± 3.53**	0.71 ± 0.15	1.87 ± 0.95
10	349 ± 14.5	85 ± 4.06*	0.72 ± 0.15	2.31 ± 1.15
A2				
0	441 ± 48.1	83 ± 12.6	1.00 ± 0.00	1.00 ± 0.00
0.1	365 ± 19.0	78 ± 9.43	0.92 ± 0.27	0.86 ± 0.07
1.0	340 ± 20.2	62 ± 9.54	0.70 ± 0.18	2.49 ± 0.30**
10	328 ± 12.7*	48 ± 2.53*	0.54 ± 0.08**	2.89 ± 0.65*
CLPr				
0	400 ± 39.5	81 ± 5.95	1.00 ± 0.00	1.00 ± 0.00
0.1	381 ± 20.3	67 ± 2.09	0.86 ± 0.08	1.12 ± 0.27
1.0	370 ± 38.3	55 ± 1.85	0.90 ± 0.13	0.82 ± 0.14
10	304 ± 15.5	48 ± 2.68	0.66 ± 0.06**	0.70 ± 0.15

[a] The data are expressed as means ± SEM ($n = 6$). Asterisks (*) denote a significant difference compared to control cultures (concentration 0): *, $p < 0.05$; **, $p < 0.01$.

DISCUSSION

The aim of the present study was to determine whether the major polyphenolic components of cacao affected ApoA1 and ApoB production in the hepatic cell line HepG2 and in the intestinal cell line Caco2. The cacao polyphenols used in this study increased ApoA1 protein and mRNA levels and decreased ApoB protein and mRNA levels in both HepG2 and Caco2 cells. These results were demonstrated by changes in protein levels in the media and mRNA levels in the cells. In addition, preincubation of HepG2 cells with each of the polyphenolic compounds enhanced LDL receptor binding activity and LDL receptor mRNA expression compared to control cultures.

Our previous study showed that daily intake of cocoa increases plasma HDL cholesterol in humans.[15] Consumption of two cups of high-polyphenol cocoa (26 g per day, 282 mg of polyphenols per day) for 12 weeks increased plasma HDL levels significantly in healthy volunteers. Furthermore, 4 weeks of ingestion of different levels of cocoa powder caused a reduction in LDL cholesterol and an elevation in HDL cholesterol levels in normocholesterolemic subjects and mildly hypercholesterolemic human volunteers.[17] Mursu et al. also reported that supplementation with high-polyphenol chocolate for 3 weeks was associated with a significant increase in serum HDL cholesterol.[14] These human studies therefore provided evidence that intake of cacao products improves cholesterol metabolism. Other studies in both rats and humans also reported that intake of polyphenols extracted from grape, tea, and soy suppressed LDL cholesterol concentrations and increased HDL cholesterol concentrations.[18−20] These results indicate that polyphenols in cocoa are responsible, at least in part, for increasing plasma HDL cholesterol concentration. Here, we elucidate part of the mechanism of the clinical antihypercholesterolemic action of cacao polyphenols.

In HepG2 cells, we observed that EC, CA, C1, A2, and CLPr increased ApoA1 protein and EC, B2, and A2 decreased Apo B protein. In Caco2 cells, we observed that EC, A2, and CLPr increased ApoA1 protein and B2, C1, and A2 decreased Apo B protein. Previous in vitro studies showed that polyphenolic substances from soy, such as genistein and daidzein, increase ApoA1[6,21] and decrease ApoB.[7] Similarly, dealcoholized red wine and the citrus flavonoids naringenin and hesperetin have been shown to decrease ApoB in these cells.[22,23] Cacao polyphenols caused a dose-dependent increase in the levels of ApoA1

Table 3. Effect of Each Cacao Polyphenol on mRNA Expression in HepG2 Cells[a]

treatment (μM)	LDLR	SR-BI	ABCA1	SREBF1	SCAP
EC					
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.1	1.14 ± 0.04**	1.05 ± 0.09	1.03 ± 0.05**	1.15 ± 0.07	1.04 ± 0.07
1.0	1.09 ± 0.10	0.97 ± 0.06	1.27 ± 0.07**	1.32 ± 0.06	0.91 ± 0.05
10	1.41 ± 0.21	1.18 ± 0.11	1.47 ± 0.13**	1.40 ± 0.14*	1.15 ± 0.16
CA					
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.1	1.29 ± 0.07**	1.18 ± 0.09	1.21 ± 0.05**	1.16 ± 0.05**	1.00 ± 0.10
1.0	1.22 ± 0.11	1.17 ± 0.12	1.29 ± 0.14	1.31 ± 0.04**	1.08 ± 0.12
10	1.44 ± 0.14*	1.45 ± 0.14**	1.59 ± 0.38	1.60 ± 0.21*	1.32 ± 0.09**
B2					
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.1	1.04 ± 0.09	0.98 ± 0.06	1.33 ± 0.16	1.25 ± 0.15	1.06 ± 0.07
1.0	1.23 ± 0.09*	0.92 ± 0.04	1.42 ± 0.22	1.23 ± 0.16	1.02 ± 0.11
10	1.39 ± 0.24	0.94 ± 0.13	2.16 ± 0.26**	1.50 ± 0.27	1.10 ± 0.18
C1					
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.1	1.06 ± 0.08	1.20 ± 0.14	0.84 ± 0.13	1.12 ± 0.08	1.01 ± 0.09
1.0	1.12 ± 0.11	0.1 ± 0.09	1.16 ± 0.11	1.23 ± 0.09*	1.21 ± 0.08*
10	1.49 ± 0.24	1.09 ± 0.14	1.39 ± 0.15*	1.43 ± 0.09**	1.37 ± 0.12*
A2					
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.1	1.51 ± 0.17*	1.17 ± 0.12	1.14 ± 0.06*	1.10 ± 0.07	1.13 ± 0.06
1.0	1.31 ± 0.36	1.00 ± 0.13	1.29 ± 0.12*	1.06 ± 0.01**	1.19 ± 0.07*
10	1.36 ± 0.15*	0.93 ± 0.09	1.55 ± 0.25*	1.35 ± 0.12*	1.36 ± 0.12*
CLPr					
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.1	1.07 ± 0.13	1.13 ± 0.08	1.03 ± 0.12	1.01 ± 0.07	1.11 ± 0.05
1.0	1.19 ± 0.14	1.15 ± 0.06*	1.27 ± 0.15	1.17 ± 0.08**	1.36 ± 0.08**
10	1.52 ± 0.14**	1.47 ± 0.11**	1.79 ± 0.34*	1.52 ± 0.18*	1.59 ± 0.17**

[a] The data are expressed as means ± SEM ($n = 6$). Asterisks (*) denote a significant difference compared to control cultures (concentration 0): *, $p < 0.05$; **, $p < 0.01$.

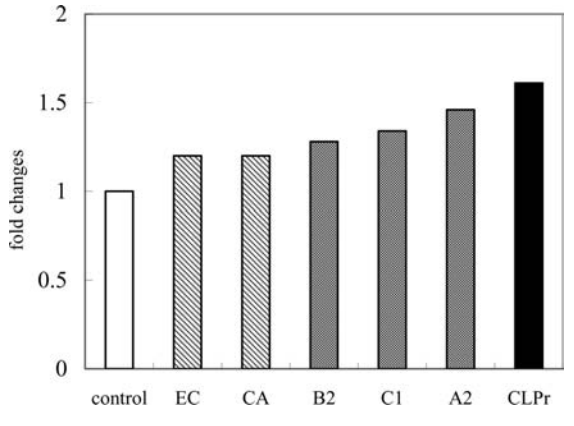

Figure 1. Effects of cacao polyphenols on LDL receptor activity in HepG2 cells. The HepG2 cells were incubated for 48 h with each cacao polyphenol at a concentration of 10 μM, followed by incubation for 5 h in Dil-labeled LDL. The level of LDL receptor activity was measured by fluorescence, with the excitation and emission wavelengths being set at 514 and 550 nm, respectively. The results were standardized to the level of fluorescence of the control cultures. Each bar represents the mean of two independent experiments.

production and a decrease in the levels of ApoB protein in both Caco2 and HepG2. Caco2 ApoA1 protein levels increased and mRNA expression levels decreased with the exception of CA and B2. Furthermore, ApoB protein levels increased and mRNA expression levels decreased with the exception of C1 and A2 in the Caco2 cells. In these cases, the increase and decrease of ApoA1/B protein levels were large. Therefore, a reversal of mRNA expression may be regulated by negative feedback.

The results of the LDL receptor binding assays and RT-PCR in the present study suggest that treatment of HepG2 cells with cacao polyphenols increases LDL receptor activity, a change that results in enhanced protein production. Treatment with either green tea polyphenols or naringenin, a citrus flavonoid, has been reported to activate LDL receptors in HepG2 cells, with these effects being mediated by SREBPs.[9,24] Cholesterol is known to regulate hepatic LDL receptor expression by activation and deactivation of transcription factors for SREBPs. The activated SREBPs then migrate to the nucleus of the cell and bind to the LDL receptor gene, resulting in increased production of protein and more cholesterol entering the cells. In the present study, treatment with cacao polyphenols increased active SREBP1 protein levels in the nuclei of the HepG2 cells. Treatment with cacao polyphenols caused similar changes in SREBP2 protein levels.

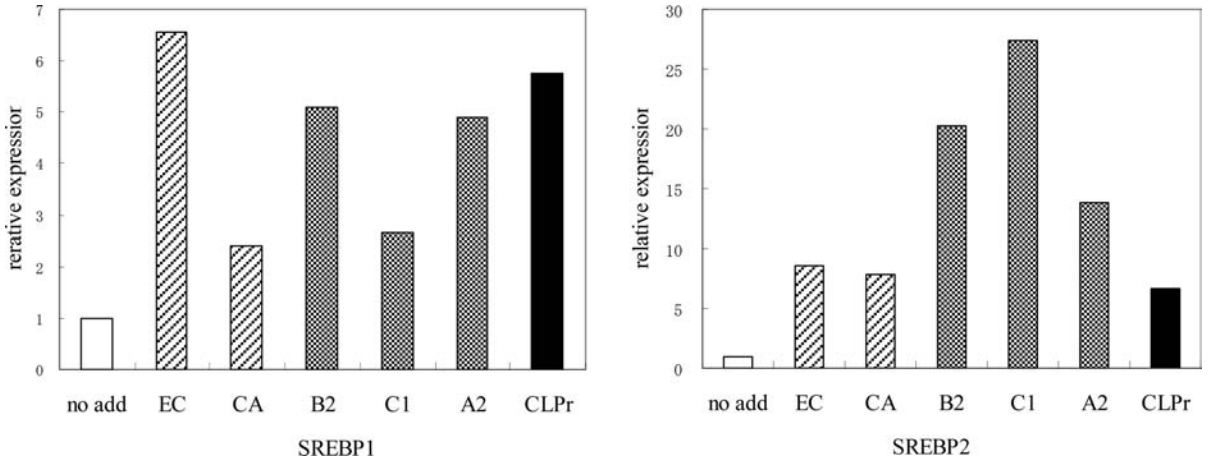

Figure 2. Effects of cacao polyphenols on activation of SREBPs in the nucleus of HepG2 cells. The HepG2 cells were incubated for 24 h with each cacao polyphenol at a concentration of 10 μM. After incubation, nuclear extracts were prepared and then separated by SDS−polyacrylamide gel electrophoresis and blotted on PVDF membranes. The SREBPs were detected using monoclonal antibodies and the relative signals determined using β-actin as a reference. Each bar represents the mean of two independent experiments.

These effects were especially apparent for B2, C1, and A2, with SREBP2 protein levels being greater than those seen with SREBP1 protein. This variability in efficacy may be attributable to differences in the chemical structure of the polyphenols. SREBP is primarily responsible for the regulation of genes involved in cholesterol biosynthesis and metabolism.[25] Therefore, these results suggest that cacao polyphenols participate in cholesterol metabolism.

In this study we also found that SR-BI mRNA expression in HepG2 cells was increased significantly by CA and CLPr (Table 3). SR-BI is a cell surface receptor that mediates selective HDL uptake[26−29] and is expressed most abundantly in liver and steroidogenic organs, where it has an important role in in vivo HDL metabolism.[29] It has been reported that treatment with testosterone up-regulates the expression of SR-BI.[28] Our results also showed that ABCA1 mRNA expression was up-regulated by all cacao polyphenols compared with control cultures. ABCA1 has a role in cholesterol efflux, with the ABCA1−apolipoprotein reaction being an important pathway in the release of cholesterol from cells and conversion to bile acid in the liver.[30] Treatment with red grape juice was shown to cause repression of ABCA1 mRNA expression in HepG2 cells,[31] whereas another study demonstrated that treatment with a flavone derivative compound activated the ABCA1 promoter in vitro and that hyperlipidemic rats treated with the same compound had significant increases in HDL cholesterol levels.[32] Treatment of macrophages with anthocyanins also resulted in activation of liver X receptors and increased ABCA1 mRNA expression. These findings suggest that anthocyanins may first activate nuclear receptors such as peroxisome proliferator activated receptor γ and liver X receptor and then, in turn, enhance ABCA1 gene expression through the transcriptional pathway.[33] In this study we did not investigate the effect of cacao polyphenol treatment on liver X receptor expression and activation, but we consider it likely that the cacao polyphenols may have a similar role in these receptors. It is therefore possible that cacao polyphenols influence ABCA1 mRNA expression by the same mechanisms as other polyphenols. Our results suggest that cacao polyphenols affect the expression of HDL cholesterol regulatory genes.

In conclusion, we investigated the effects of cacao polyphenols on ApoA1 and ApoB production in the human hepatoma cell line HepG2 and the human intestinal cell line Caco2. Treatment with cacao polyphenols increased ApoA1 and decreased ApoB production in both cell lines. These effects were the result of up-regulation of mature form SREBPs and increased LDL receptor activity induced by the cacao polyphenols. We have therefore identified the mechanism responsible for the elevation in HDL cholesterol associated with daily cocoa intake. As cholesterol metabolism is known to be regulated by several different mechanisms, it is possible that cacao polyphenols may act on multiple pathways as a regulatory receptor agonist or ligand, similar to other plant polyphenols.

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REFERENCES

(1) Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **1989**, *320*, 915–924.

(2) Gordon, D. J.; Probstfield, J. L.; Garrison, R. J.; Neaton, J. D.; Castelli, W. P.; Knoke, J. D.; Jacobs, D. R., Jr.; Bangdiwala, S.; Tyroler, H. A. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* **1989**, *79*, 8–15.

(3) Arts, I. C. W.; Hollman, P. C. H.; Feskens, E. J. M.; Bueno de Mesquita, H. B.; Kromhout, D. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *Am. J. Clin. Nutr.* **2001**, *74*, 227–232.

(4) Arts, I. C. W.; Jacobs, D. R., Jr.; Harnack, L. J.; Gross, M.; Folsom, A. R. Dietary catechins in relation to coronary heart disease death among postmenopausal women. *Epidemiology* **2001**, *12*, 668–675.

(5) Goodman-Gruen, D.; Kritz-Silverstein, D. Usual dietary isoflavone intake is associated with cardiovascular disease risk factors in postmenopausal women. *J. Nutr.* **2001**, *131*, 1202–1206.

(6) Lamon-Fava, S.; Micherone, D. Regulation of apoA-I gene expression: mechanism of action of estrogen and genistein. *J. Lipid Res.* **2004**, *45*, 106−112.

(7) Borradaile, N. M.; de Dreu, L. E.; Wilcox, L. J.; Edwards, J. Y.; Huff, M. W. Soya phytoestrogens, genistein and daidzein, decrease apolipoprotein B secretion from HepG2 cells through multiple mechanisms. *Biochem. J.* **2002**, *366*, 531–539.

(8) Borradaile, N. M.; de Dreu, L. E.; Barrett, P. H.; Huff, M. W. Inhibition of hepatocyte apoB secretion by naringenin: enhanced rapid intracellular degradation independent of reduced microsomal cholesteryl esters. *J. Lipid Res.* **2002**, *43*, 1544–1554.

(9) Borradaile, N. M.; de Dreu, L. E.; Huff, M. W. Inhibition of net HepG2 cell apolipoprotein B secretion by the citrus flavonoid naringenin involves activation of phosphatidylinositol 3-kinase, independent of insulin receptor substrate-1 phosphorylation. *Diabetes* **2003**, *52*, 2554–2561.

(10) Allister, E. M.; Borradaile, N. M.; Edwards, J. Y.; Huff, M. W. Inhibition of microsomal triglyceride transfer protein expression and apolipoprotein B100 secretion by the citrus flavonoid naringenin and by insulin involves activation of the mitogen-activated protein kinase pathway in hepatocytes. *Diabetes* **2005**, *54*, 1676–1683.

(11) Takechi, R.; Hiramatsu, N.; Mamo, J. C.; Pal, S. Red wine polyphenolics suppress the secretion and the synthesis of Apo B48 from human intestinal CaCo-2 cells. *Biofactors* **2004**, *22*, 181–183.

(12) Pal, S.; Ho, N.; Santos, C.; Dubois, P.; Mamo, J.; Croft, K.; Allister, E. Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *J. Nutr.* **2003**, *133*, 700–706.

(13) Osakabe, N.; Yasuda, A.; Natsume, M.; Takizawa, T.; Terao, J.; Kondo, K. Catechins and their oligomers linked by C4 → C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. *Exp. Biol. Med. (Maywood)* **2002**, *227*, 51–56.

(14) Mursu, J.; Voutilainen, S.; Nurmi, T.; Rissanen, T. H.; Virtanen, J. K.; Kaikkonen, J.; Nyyssonen, K.; Salonen, J. T. Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. *Free Radical Biol. Med.* **2004**, *37*, 1351–1359.

(15) Baba, S.; Osakabe, N.; Kato, Y.; Natsume, M.; Yasuda, A.; Kido, T.; Fukuda, K.; Muto, Y.; Kondo, K. Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans. *Am. J. Clin. Nutr.* **2007**, *85*, 709–717.

(16) Natsume, M.; Osakabe, N.; Yamagishi, M.; Takizawa, T.; Nakamura, T.; Miyatake, H.; Hatano, T.; Yoshida, T. Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal- phase and reversed-phase HPLC. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 2581–2587.

(17) Baba, S.; Natsume, M.; Yasuda, A.; Nakamura, Y.; Tamura, T.; Osakabe, N.; Kanegae, M.; Kondo, K. Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. *J. Nutr.* **2007**, *137*, 1436–1441.

(18) Kajimoto, O.; Kajimoto, Y.; Yabune, M.; Nozawa, A.; Nagata, K.; Kakuda, T. Tea catechins reduce serum cholesterol levels in mild and borderline hypercholesterolemia patients. *J. Clin. Biochem. Nutr.* **2003**, *33*, 101–111.

(19) Yamakoshi, J.; Kataoka, S.; Koga, T.; Ariga, T. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **1999**, *142*, 139–149.

(20) Zhan, S.; Ho, S. C. Meta-analysis of the effects of soy protein containing isoflavones on the lipid profile. *Am. J. Clin. Nutr.* **2005**, *81*, 397–408.

(21) Lamon-Fava, S. Genistein activates apolipoprotein A-I gene expression in the human hepatoma cell line Hep G2. *J. Nutr.* **2000**, *130*, 2489–2492.

(22) Pal, S.; Ho, N.; Santos, C.; Dubois, P.; Mamo, J.; Croft, K.; Allister, E. Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *J. Nutr.* **2003**, *133*, 700–706.

(23) Wilcox, L. J.; Borradaile, N. M.; de Dreu, L. E.; Huff, M. W. Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, via reduced activity and expression of ACAT2 and MTP. *J. Lipid Res.* **2001**, *42*, 725–734.

(24) Bursill, C.; Roach, P. D.; Bottema, C. D.; Pal, S. Green tea up-regulates the low-density lipoprotein receptor through the sterol-regulated element binding Protein in HepG2 liver cells. *J. Agric. Food Chem.* **2001**, *49*, 5639–5645.

(25) Horton, J. D.; Goldstein, J. L.; Brown, M. S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **2002**, *109*, 1125–1131.

(26) Sahoo, D.; Peng, Y.; Smith, J. R.; Darlington, Y. F.; Connelly, M. A. Scavenger receptor class B, type I (SR-BI) homo-dimerizes via its C-terminal region: fluorescence resonance energy transfer analysis. *Biochim. Biophys. Acta* **2007**, *1771*, 818–829.

(27) Rigotti, A.; Edelman, E. R.; Seifert, P.; Iqbal, S. N.; DeMattos, R. B.; Temel, R. E.; Krieger, M.; Williams, D. L. Regulation by adrenocorticotropic hormone of the in vivo expression of scavenger receptor class B type I (SR-BI), a high density lipoprotein receptor, in steroidogenic cells of the murine adrenal gland. *J. Biol. Chem.* **1996**, *271*, 33545–33549.

(28) Langer, C.; Gansz, B.; Goepfert, C.; Engel, T.; Uehara, Y.; von Dehn, G.; Jansen, H.; Assmann, G.; von Eckardstein, A. Testosterone up-regulates scavenger receptor BI and stimulates cholesterol efflux from macrophages. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 1051–1057.

(29) Brundert, M.; Heeren, J.; Bahar-Bayansar, M.; Ewert, A.; Moore, K. J.; Rinninger, F. Selective uptake of HDL cholesteryl esters and cholesterol efflux from mouse peritoneal macrophages independent of SR-BI. *J. Lipid Res.* **2006**, *47*, 2408–2421.

(30) Tsujita, M.; Wu, C. A.; Abe-Dohmae, S.; Usui, S.; Okazaki, M.; Yokoyama, S. On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway. *J. Lipid Res.* **2005**, *46*, 154−162.

(31) Davalos, A.; Fernandez-Hernando, C.; Cerrato, F.; Martinez-Botas, J.; Gomez-Coronado, D.; Gomez-Cordoves, C.; Lasuncion, M. A. Red grape juice polyphenols alter cholesterol homeostasis and increase LDL-receptor activity in human cells in vitro. *J. Nutr.* **2006**, *136*, 1766–1773.

(32) Guo, L.; Hu, W. R.; Lian, J. H.; Ji, W.; Deng, T.; Qian, M.; Gong, B. Q. Anti-hyperlipidemic properties of CM108 (a flavone derivative) in vitro and in vivo. *Eur. J. Pharmacol.* **2006**, *551*, 80–86.

(33) Xia, M.; Hou, M.; Zhu, H.; Ma, J.; Tang, Z.; Wang, Q.; Li, Y.; Chi, D.; Yu, X.; Zhao, T.; Han, P.; Xia, X.; Ling, W. Anthocyanins induce cholesterol efflux from mouse peritoneal macrophages: the role of the peroxisome proliferator-activated receptor γ-liver X receptor α-ABCA1 pathway. *J. Biol. Chem.* **2005**, *280*, 36792–36801.