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Cruciferous Indole-3-Carbinol Inhibits Apolipoprotein B Secretion in HepG2 Cells[1–3]

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Abstract

The cardioprotective effect of consuming cruciferous vegetables may be attributed to a number of unique indole-based compounds. We investigated the potential role and mechanism of action of an indole-based compound, indole-3-carbinol (I-3-C), on apolipoprotein B-100 (apoB) production using HepG2 cells. I-3-C reduced apoB secretion into the media dose dependently by 56% at 100 μmol/L. Relative to the untreated control cells, no change in the density of the secreted lipoproteins was noted. Significant decreases in cellular lipid synthesis, including triglycerides (TG) and cholesterol esters (CE), were observed in cells treated with I-3-C, indicating that limited lipid availability is a major factor in the regulation of apoB secretion. The decrease in TG synthesis was associated with significantly decreased diacylglycerol acyltransferase-1 and -2 activity and reduced fatty acid synthase (FASN) gene expression. The decreased CE synthesis was associated with significantly decreased acyl CoA:cholesterol acyltransferase gene expression and activity. The effect on FASN was shown to be mediated by sterol regulatory element binding protein-1, an important transcription factor involved in fatty acid synthesis. Further investigative work revealed that LDL uptake and fatty acid oxidation were not involved in the I-3-C-mediated reduction of apoB secretion. The results indicate that plant indoles have beneficial effects on lipid synthesis that could contribute to their potential cardioprotective effect. J. Nutr. 137: 2185–2189, 2007.

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Introduction

Dietary intake of fruits and vegetables has been investigated extensively in the prevention of cardiovascular disease (CVD),[6] a leading cause of mortality in the United States. Increasing evidence has indicated that people who consume more fruits and vegetables have a lower prevalence of important risk factors for CVD, including hypertension, obesity, and type 2 diabetes [reviewed in (1,2)]. When these foods were further analyzed, cruciferous vegetables interestingly conferred the greatest protection [reviewed in (2)]. Cruciferous vegetables include broccoli, cabbage, cauliflower, brussel sprouts, turnip, and radish (3). Wu et al. (4) recently demonstrated that broccoli sprouts given to hypertensive stroke-prone rats protected against CVD by attenuating oxidative stress, hypertension, and inflammation. A possible explanation for the enhanced protection may be that cruciferous vegetables are particularly rich in indolic compounds, namely glucosinolates (GS) and phytoalexins (5). GS and phytoalexins are secondary plant metabolites that are thought to function in the microbial/fungal defense of crucifers (3,5). GS share a basic chemical structure consisting of a β-D-thioglucose group, a sulfonated oxime group, and a side chain derived from methionine, phenylalanine, tryptophan, or branched-chain amino acids. More than 120 different GS have been characterized from plants. Those derived from tryptophan are classified as indole GS. GS are not bioactive until they are enzymatically hydrolyzed upon maceration (i.e. chewing, food preparation) to various metabolites (i.e. isothiocyanates, thiocyanates, and nitriles), which, following ingestion, are subject to further metabolism (3). Crucifers also contain phytoalexins, an indole derivative resembling indole GS (5). Dunn et al. (6) reported over a decade ago that indole-3-carbinol (I-3-C), a plant indole GS common to cruciferous vegetables, reduced serum cholesterol levels in a hypercholesterolemic mice. The lipogenic enzyme, acyl CoA: cholesterol acyltransferase (ACAT), an enzyme involved in cholesterol esterification essential for lipoprotein assembly, was shown to be the mechanism responsible for lowering cholesterol levels (6). These observations were pivotal in pursuing our study. We sought to examine the effect of I-3-C on the secretion of apolipoprotein B-100 (apoB), the major structural component of VLDL and LDL, using the well-established human hepatoma

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[3] Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.
[6] Abbreviations used: ACAT, acyl CoA:cholesterol acyltransferase; apoB, apolipoprotein B-100; apoB-Lp, apoB-containing lipoprotein; CE, cholesterol ester; CVD, cardiovascular disease; CPT, carnitine palmityol transferase; DGAT, diacylglycerol acyltransferase; DiI, 3,3′-dioctadecylindocarbocyanine; DMSO, dimethyl sulfoxide; FASN, fatty acid synthase; GS, glucosinolates; HMGR, hydroxymethyl CoA reductase; I-3-C, indole-3-carbinol; LPDS, lipoprotein deficient serum; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein; SREBP, sterol regulatory element binding protein; TG, triglyceride.
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cell line, HepG2, as the model system. Additional studies were undertaken to define the regulation of apoB secretion by I-3-C. Because GS and phytoalexins are important micronutrients in cruciferous vegetables, this study could contribute to a better understanding of these molecules in the treatment of hyperlipidemia.

Materials and Methods

Cell culture. HepG2 cell cultures (HB 8065; American Type Culture Collection) were maintained in RPMI-1640 medium with 10% fetal bovine serum (Invitrogen) and subcultured in 35-mm-diameter dishes to ~80% confluence. Cells were treated with I-3-C for 24 h in RPMI medium containing 5% human lipoprotein-deficient serum (LPDS). LPDS was prepared in-house by discontinuous density gradient ultracentrifugation using KBr. I-3-C (>99% purity; LKT Laboratories) was solubilized in dimethyl sulfoxide (DMSO). An appropriate amount of stock solution was diluted in culture medium to give a final DMSO concentration in the dishes of ≤0.25%. Stock solutions were kept at −20°C for no longer than 4 wk. Control cells were treated with the solvent (i.e. DMSO) only.

ApoB ELISA. ApoB secreted into the medium was determined using a noncompetitive binding ELISA procedure as described by Casaschi et al. (7). Spectrophotometric readings were normalized to cell protein.

Lipoprotein fractionation. Cells were treated for 22 h and pulse labeled with protein labeling medium (100 μCi/mL [^{35}S]protein labeling mix in LPDS-RPMI ± I-3-C) for 2 h essentially as described by Theriault et al. (8) with minor modifications. Conditioned media was subjected to ultracentrifugation in a KBr gradient according to the method described by Tovar et al. (9). Media was fractionated into 12 fractions and subjected to immunoprecipitation of apoB, SDS-PAGE, and fluorography. ApoB radioactivity was quantified by scintillation counting of the apoB band in the dried gel. The density was measured in each fraction. Fractions 3–8 represent dense LDL apoB-containing lipoprotein (apoB-Lp), whereas fractions 8–12 represent the HDL-like apoB-Lp.

Lipid synthesis and secretion. Free cholesterol, triglyceride (TG), and cholesterol ester (CE) synthesis and secretion was measured as described by Casaschi et al. (10) with minor modifications. To measure cholesterol, treated and untreated cells were labeled with [^{3}H]acetate (10 mCi/L, 3 Ci/mmol, Perkin Elmer Life Science Research Products). For TG and CE, cells were labeled with [^{3}H]oleic acid (10 mCi/L, 23 Ci/mmol, Perkin Elmer Life Science Research Products). Labeling occurred in the final 6 h of the 24-h treatment period.

Real-time RT-PCR. Total RNA was isolated using RNA-Bee Isolation Reagent (Tel-Test) using the manufacturer's protocol. Four micrograms of total RNA was converted to first-strand cDNA in a single reaction volume (20 μL) using Superscript II RT and random primers (Invitrogen) according to the manufacturer's instructions. For real time PCR, each replicate contained 1 μL template, 2 μL 10 μmol/L primers, and 22 μL water to which 25 μL of iQ SYBR Green Supermix (Biorad Laboratories) was added. Duplicate reactions were conducted for each sample on a MyiQ Thermal Cycler (Biorad Laboratories). The PCR profile was initiated with a 10-min denaturation at 95°C, followed by 40 cycles of a 30-s denaturation at 95°C, and a 30-s combined annealing/extension step at 60°C. Expression of each gene was normalized to hypoxanthine-guanine phosphoribosyltransferase expression for each sample. The genes, primer sequences, and sources used in this study are in **Supplemental Table 1**.

Enzyme activity. Diacylglycerol acyltransferase (DGAT)-1 and -2 activity was measured in whole cells as described by Casaschi et al. (11) using [^{14}C]palmitoyl-CoA with minor modifications. The experiments were conducted in the presence of 10 mmol/L and 100 mmol/L of $MgCl_2$. DGAT-2 activity is known to be inhibited by high concentrations of $MgCl_2$ (12), allowing DGAT-2 activity to be calculated by subtracting the value obtained at 100 mmol/L $MgCl_2$ from the value obtained at 10 mmol/L $MgCl_2$. ACAT activity from isolated microsomes (50 μg) was determined using [^{14}C]palmitoyl-CoA according to the method described by Jeon et al. (13). Microsomal TG transfer protein (MTP) activity in cell extracts was measured by a fluorescent assay according to the manufacturer's protocol (Roar Biomedical).

LDL uptake. Human LDL labeled with the fluorescent probe 3,3′-dioctadecylindocarbocyanine (DiI) was purchased from Biomedical Technologies. LDL uptake in whole cells was performed as described by Stephan and Yurachek (14). Briefly, treated and untreated cells were incubated with 30 mg/L of DiI-LDL at 37°C for 2 h. Cells were washed with PBS and isopropanol was added to each well. The isopropanol extract was transferred to glass tubes and centrifuged at 3500 × *g*; 10 min and the fluorescence was determined using a spectrofluorometer (Photon Technology International). DiI content in the extracts was calculated from a DiI standard curve. Values were expressed as micrograms of DiI-LDL protein per milligram of cell protein.

Western blot. Protein expression levels of the mature forms of sterol regulatory element binding protein (SREBP)-1 and -2 were determined by extracting nuclear proteins from cell lysates as previously described (15). Nuclear proteins (40 μg) were separated by SDS-PAGE and immunoblotted using mouse monoclonal antibodies against SREBP-1 (2A4) or SREBP-2 (1C6) obtained from Santa Cruz Biotechnology. The corresponding protein band was quantified by densitometry (BioRad Gel Doc 2000 system).

Other methods. Cell protein content was measured according to Bradford (16) (i.e. Bio-Rad) using bovine serum albumin as the standard. Albumin secreted into the medium was performed using an ELISA kit from Bethyl Laboratories.

Statistical analysis. Statistical analysis was conducted using Student's *t* tests for 2-group comparisons. For multiple comparisons, 1-way ANOVA followed by a Student-Newman-Keuls test was used. Values are expressed as means ± SD and $P < 0.05$ was considered significant.

Results

I-3-C inhibits apoB secretion. I-3-C treatment in cells under lipid-depleted condition reduced apoB secretion in a dose-dependent manner after 24 h of incubation as assayed by ELISA (**Fig. 1**). I-3-C did not affect albumin secretion except at the highest dose of 200 μmol/L, indicating the effects on apoB secretion below the 200 μmol/L concentration are specific and a concentration of 200 μmol/L or greater may be indicative of cell toxicity. Consequently, we chose 50 μmol/L as the optimal concentration for use in the subsequent experiments. Using pulse-labeling and fractionation techniques, cells incubated with I-3-C secreted remarkably fewer apoB-Lp particles but with a density distribution pattern that was similar to the untreated control cells (**Fig. 2**).

I-3-C decreases lipid synthesis and secretion. Synthesis of cellular TG and CE decreased significantly in the presence of I-3-C (54 ± 16%, 52 ± 15%, respectively, $P < 0.05$ vs. control; **Fig. 3**). I-3-C treatment also significantly reduced the secretion of TG (52 ± 16%) and free cholesterol (38 ± 15%) ($P < 0.05$ vs. control).

I-3-C inhibits lipogenesis. The decreased TG synthesis was associated with significantly decreased DGAT-1 (56%) and -2 activity (59%) ($P < 0.05$ vs. control; **Table 1**) and reduced fatty acid synthase (FASN) gene expression (25 ± 8%; $P < 0.05$ vs. control; **Fig. 4**). The decreased CE synthesis was associated with significantly decreased ACAT gene expression (34 ± 8%;

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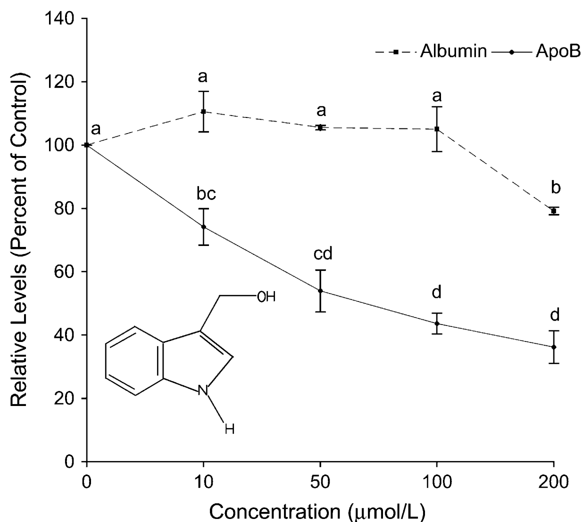

FIGURE 1 Effect of I-3-C on apoB and albumin secretion in HepG2 cells. Cells were treated with various concentrations of I-3-C (10–200 μmol/L) for 24 h in 5% LPDS-RPMI medium. The medium was collected and apoB/albumin secretion was measured by ELISA. Data are expressed as a percentage of control (set as 100%). Values are means ± SD of 3 independent experiments performed in duplicate ($n = 3$). Means for a protein without a common letter differ, $P < 0.05$. Insert: Structure of I-3-C.

$P < 0.05$ vs. control; Fig. 4) and activity level (23%; $P < 0.05$ vs. control; Table 1). No significant effect on HMG-CoA reductase (HMGR) gene expression was observed ($P = 0.52$ vs. control; data not shown).

I-3-C decreases expression of SREBP-1 and-2, key modulators of lipid synthesis. I-3-C significantly inhibited the protein expression of the mature form of SREBP-1 and SREBP-2 present in the nucleus by 44 ± 9% and 37 ± 15%, respectively, ($n = 3$, $P < 0.05$ vs. control; data not shown). A representative immunoblot showing the signals corresponding to the nuclear forms of SREBP-1 and -2 from 1 of these experiments is shown in **Figure 5**. SREBP-1c gene expression was also reduced by 38 ± 9% ($P < 0.01$ vs. control; Fig. 4), whereas SREBP-2 tended to be lower than in the control ($P = 0.07$; Fig. 4).

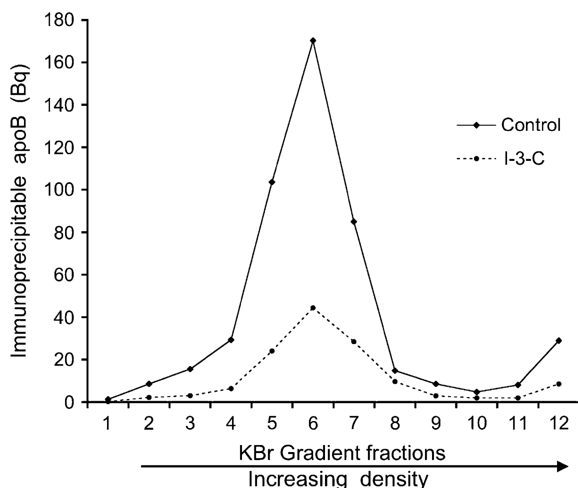

FIGURE 2 Density distribution of secreted apoB-Lp in HepG2 cells. Cells were treated with I-3-C (50 μmol/L) for a total of 24 h. Values represent a single fractionation sample of a representative experiment (reproduced in 2 other experiments).

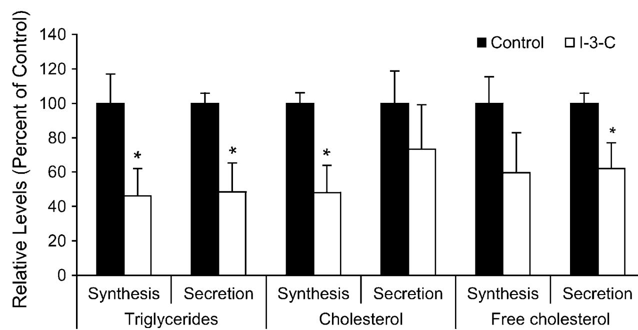

FIGURE 3 Effect of I-3-C on lipid synthesis and secretion in HepG2 cells. Cells were treated as in Figure 2. Results are expressed as a percentage of untreated control (set at 100%). Values are means ± SD of 3 independent experiments performed in triplicate. *Different from control, $P < 0.05$.

I-3-C inhibits lipid transfer. A significant reduction in the activity level of MTP, an enzyme involved in lipid transfer to the primordial apoB-Lp, was observed in cells incubated in the presence of I-3-C (Table 1; 17%; $P < 0.05$ vs. control).

I-3-C has no effect on LDL uptake. The effect of I-3-C (50 μmol/L) did not affect cellular uptake of DiI-LDL or, thus, LDL receptor activity compared with the untreated control cells (0.67 ± 0.02 and 0.71 ± 0.01 μg/mg cell protein, respectively; $P = 0.97$ vs. control, $n = 3$).

I-3-C has no effect on fatty acid oxidation. Gene expression of carnitine palmityol transferase-1 (CPT-1), an enzyme involved in fatty acid oxidation, showed no effect compared with the untreated control cells ($P = 0.37$ vs. control; data not shown). The mRNA levels of PPARα, the transcription factor with a positive regulatory impact on CPT-1 gene expression, were also not affected ($P = 0.44$ vs. control; data not shown).

Discussion

Most of the clinical and experimental studies on cruciferous vegetables and related indole compounds have concentrated on its anticarcinogenic property (3,17) . Fewer studies, on the other hand, have examined the effect of cruciferous vegetable-rich diets and, particularly, indole GS, on risk factors associated with CVD. Despite some studies linking increased cruciferous vegetable and indole GS intakes to CVD and stroke prevention (2,4,6), evidence of this role has been sparse and the biological mechanism for this benefit remains essentially unexplored.

Our results provide further evidence of and the mechanism for the lowering of apoB-Lp secretion by indole GS. Treatment of HepG2 cells with pharmacological concentrations of I-3-C was shown to be a potent inhibitor of apoB secretion. I-3-C treatment inhibited apoB secretion in a dose-dependent manner and the effect was specific, as levels of another secreted protein (i.e., albumin) remained essentially unchanged up to 100 μmol/L. Using pulse labeling and lipoprotein fractionation experiments, a remarkable reduction in radiolabeled apoB in the medium was observed when cells were treated with 50 μmol/L I-3-C relative to untreated control. The secreted apoB molecule was associated predominantly with an LDL-sized particle and showed no shift in the density distribution when compared with

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TABLE 1 Lipogenic enzyme activities in control and I-3-C treated HepG2 cells[1,2]

Treatment	DGAT-1	DGAT-2	ACAT	MTP
	nmol [^{14}C]TG·min^{-1}·mg cell protein^{-1}	*nmol [^{14}C]TG·min^{-1}·mg cell protein^{-1}*	*pmol [^{14}C]CH·min^{-1}·mg cell protein^{-1}*	*fluorescence intensity transferred/mg cell protein × 10^{-6}*
Control	2.3 ± 0.1	4.7 ± 0.5	95.8 ± 3.7	4.5 ± 0.2
I-3-C	1.0 ± 0.2*	1.9 ± 0.1*	74.5 ± 4.2*	3.7 ± 0.1*

[1] Values are means ± SD of 3 independent experiments performed in duplicate. *Different from untreated control, $P < 0.05$.
[2] Cells were treated with 50 μmol/L of I-3-C for 24 h.

untreated control cells. This indicates that I-3-C inhibits apoB secretion without altering the lipid composition of the secreted lipoprotein. This is the first report to our knowledge to indicate that plant indoles possess the ability to decrease the number of apoB-Lp secreted.

The availability of lipid is a major determining factor in the regulation of apoB assembly and secretion (18). It plays a central role in apoB targeting, either for intracellular degradation or for assembly as lipoprotein particles. Lipid availability in the liver can be modulated by 3 metabolic pathways: lipid synthesis, LDL uptake, and fatty acid oxidation.

To examine the mechanism underlying the reduction in hepatic apoB secretion by I-3-C, we first examined its effect on lipid synthesis. A reduction in lipid synthesis by I-3-C would potentially reduce the amount of lipid substrates required for the assembly of apoB-Lp, resulting in the reduction of apoB secretion. Interestingly, significant decreases in cellular lipid synthesis, including TG and CE, were observed. This inhibition was in agreement with the observations made on the effect of I-3-C treatment on key lipogenic genes, including DGAT, FASN, and ACAT, as determined by PCR and enzyme activity assays. I-3-C significantly decreased the gene and protein expression level of SREBP-1, a transcription factor that is involved in the regulation of fatty acid synthesis (19). This effect was confirmed by the reduction of its downstream target gene, FASN, a key enzyme in fatty acid synthesis. A notable reduction was also observed on the gene and protein expression level of SREBP-2, which regulates cholesterol homeostasis. However, the gene expression level of HMGR, the rate-limiting enzyme in cholesterol synthesis and a downstream target gene of SREBP-2, remained unchanged. The reason for the discrepancy is not known; however, the effects on HMGR are consistent with the results on cholesterol synthesis, which did not reach significance.

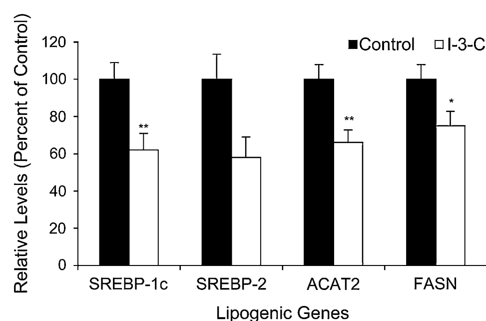

FIGURE 4 Effect of I-3-C on key lipogenic gene expression in HepG2 cells. Cells were treated as in Figure 2. Total RNA was isolated and levels of messages were quantitated by real-time PCR. Gene expression levels in treated cells are relative to levels in untreated control cells set at 100%. Data are means ± SEM of 3 samples of a single experiment. Amplification reactions were conducted in duplicate for each gene. Asterisks indicate different from control, $*P < 0.05$; $**P < 0.01$.

The regulation of SREBP-1c expression has been shown to occur dependently and independently of liver X receptor (LXR)α (20). We sought to determine whether the suppression in the gene/protein expression of SREBP-1 with I-3-C treatment was mediated by LXRα. The expression level of LXRα mRNA levels in the presence and absence of I-3-C treatment did not differ, indicating that I-3-C acts either directly on SREBP-1c or via a pathway independent of LXRα.

Lipogenic enzymes independent of SREBP regulation such as ACAT and DGAT were also shown to be modulated by I-3-C. Cells treated with I-3-C showed a moderate reduction in the gene expression and activity level of ACAT, an enzyme involved in cholesterol esterification essential for lipoprotein assembly. This confirms previous work done on ACAT and I-3-C (21). The gene expression level of DGAT1 and DGAT2, key enzymes in TG synthesis (11), remained unchanged, but their activity levels were significantly reduced. The discrepancy may be explained by the fact that DGAT is thought to be regulated primarily at the post-transcriptional level (22). Among all of the lipogenic enzymes tested, DGAT was inhibited the greatest. The greater inhibition on DGAT compared with ACAT may be a reflection that TG, and not CE, is considered the primary lipid constituent in the regulation of apoB secretion under our experimental condition. Together, these findings support the hypothesis that I-3-C may reduce the amount of lipid substrates available for the assembly of apoB-Lp by inhibiting their biosynthesis.

In addition to lipid synthesis, LDL uptake and fatty acid oxidation are also involved in the maintenance of lipid homeostasis in the liver. In regards to LDL uptake, no significant effect on cellular uptake of fluorescent labeled LDL was observed with I-3-C treatment. This coincided with no changes on LDL receptor gene expression, confirming that reuptake of apoB-Lp does not explain the diminished apoB accumulation in the media. In the case of fatty acid oxidation, no effects were observed on gene expression of CPT-1, an enzyme involved in fatty acid oxidation, and PPARα RNA levels, the transcription factor with a positive regulatory impact on CPT-1 gene expression.

Evidence has shown that aside from lipid availability, lipid transfer to the nascent apoB molecule to form the primordial lipoprotein is also required for the secretion of apoB-Lp (23). Together with lipid availability, both actions are considered major determining factors in the assembly and secretion of apoB-Lp.

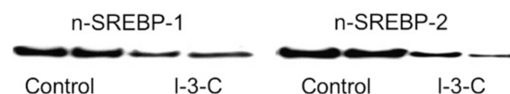

FIGURE 5 Effect of I-3-C on SREBP protein expression in HepG2 cells. Cells were treated as in Figure 2. Figure is a representative immunoblot showing the signals corresponding to SREBP-1 and -2 in the nuclear (n) extracts (mature form). Reproduced in 2 other experiments in duplicate.

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We therefore continued our investigations by examining the activity level of MTP, the enzyme involved in lipid transfer to apoB. I-3-C significantly decreased MTP activity, but the reduction was rather minimal.

In conclusion, this study indicated that I-3-C is a potent inhibitor of hepatic apoB secretion. Modulation of cellular lipid synthesis was the primary factor in the regulation of apoB secretion. Reduced lipid synthesis via SREBP-1 and its downstream gene, FASN, was one of the mechanisms for the suppression of apoB-Lp secretion by I-3-C. Other lipogenic enzymes independent of SREBP regulation, including DGAT and ACAT, were also involved. Together, plant indoles resulted in beneficial effects on lipid metabolism that could contribute to their potential cardioprotective effect.

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