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HYPOCHOLESTEROLEMIC PROPERTIES OF PLANT INDOLES

INHIBITION OF ACYL-CoA:CHOLESTEROL ACYLTRANSFERASE ACTIVITY AND REDUCTION OF SERUM LDL/VLDL CHOLESTEROL LEVELS BY GLUCOBRASSICIN DERIVATIVES

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Abstract—Studies were undertaken to investigate the effects of the plant compound indole-3-carbinol (I3C) and its acid condensation products, which are generated in the stomach following ingestion of I3C, on cholesterol homeostasis in mice. Individual acid condensation products were synthesized and purified by HPLC. *In vitro* experiments revealed that several of the acid condensation products effectively inhibited the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), which is responsible for the conversion of free cholesterol to the cholesteryl ester, at micromolar concentrations. Since the inhibition of ACAT *in vivo* should reduce serum cholesterol levels, I3C was administered to mice, and the effects on serum cholesterol levels were evaluated. Total serum cholesterol levels were elevated by 29% in mice provided a 3% cholesterol-supplemented diet, but this elevation was attenuated significantly ($P \leq 0.05$) by approximately 50% when I3C (100 mg/kg/day) was added to this diet. This effect of I3C was entirely on low density lipoprotein (LDL)/very low density lipoprotein (VLDL) cholesterol, which was lowered significantly ($P \leq 0.05$) by approximately 30%. In summary, I3C lowered serum LDL/VLDL cholesterol levels in mice, and this effect was likely mediated by the inhibition of ACAT by some of the acid condensation products of I3C. These results provide a possible mechanism by which I3C-rich vegetables lower serum cholesterol levels.

Key words: indole-3-carbinol, cholesterol, acyl-CoA:cholesterol acyltransferase

Atherosclerosis is the leading cause of death in developed western societies, causing such maladies as cardiovascular disease, stroke, and peripheral vascular disease [1]. Cardiovascular disease alone accounts for over half a million deaths in the United States annually [2]. A major risk factor associated with the development of atherosclerosis is high blood cholesterol associated with low density lipoprotein (LDL)† [3–5]. Once cholesterol, derived from either diet or enterohepatic recirculation, is absorbed into the epithelial cells of the intestine, it undergoes esterification with long chain fatty acids by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT). The resulting cholesteryl ester is then available for incorporation into chylomicrons and transport to the liver [6–8]. Cholesteryl ester is de-esterified in the parenchymal cells of the liver by the enzyme cholesterol ester hydrolase [9]. This free cholesterol can be eliminated from the body via metabolic conversion to bile acids or directly in association with bile acid/phosphatidylcholine micelles [10–12]. Alternatively, the free cholesterol can be re-esterified by hepatic ACAT and the cholesteryl esters can be stored in the cytoplasm of the liver as lipid droplets or incorporated into lipoproteins for systemic distribution. ACAT inhibition thus provides a potential mechanism by which blood cholesterol levels can be lowered since inhibition of intestinal ACAT would impede the uptake of cholesterol, and inhibition of hepatic ACAT would enhance the free cholesterol pool that is available for elimination. In light of this pivotal role of ACAT in the uptake and elimination of cholesterol, significant effort has been expended in recent years to identify hypocholesterolemic ACAT-inhibiting drugs [6, 13–16].

Glucobrassicin is a secondary plant metabolite that is abundant in cruciferous vegetables of the genus *Brassica* (e.g. cabbage, broccoli, and cauliflower) [17]. Glucobrassicin undergoes autolysis during maceration (e.g. chewing, food preparation) to indole-3-carbinol (I3C), which, following ingestion, is subject to acid condensation in the stomach yielding various oligomeric derivatives. Several of these acid condensation products have been detected in the small intestine and liver of rats following oral administration of I3C [18, 19]. The ACAT-inhibiting hypocholesterolemic drug Bay o 2752 [*N*,*N*′-(1,11-undecandiyl)bis-2,3-dihydro-2-methyl-1*H*-indole-1-carboxamide] also has a multi-indolic structure [14],

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† Abbreviations: LDL, low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; I3C, indole-3-carbinol; DIM, 3,3′-diindolylmethane; CTI, 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole; BII, 2,3-bis[3-indolylmethyl]indole; VLDL, very low density lipoprotein; HDL, high density lipoprotein; and DMSO, dimethyl sulfoxide.

and we considered the possibility that some acid condensation products of I3C may similarly inhibit ACAT activity in the intestine and/or liver following ingestion of I3C. Such activity may provide a biochemical mechanism by which consumption of cruciferous vegetables could lower blood cholesterol levels.

Several lines of evidence suggest the presence of hypocholesterolemic compounds in some vegetables. Epidemiological studies have shown that populations having diets high in vegetables have lower serum cholesterol levels and reduced incidences of coronary heat disease [20–22]. This observation is not due entirely to the consumption of less animal products in such populations since clinical studies have shown that increased vegetable consumption along with a normal caloric diet lowers serum cholesterol levels [23]. Specifically, glucobrassicin-rich rape seed oil, when administered along with a normal diet to hypercholesterolemic individuals, reduced serum cholesterol levels by 8.5% [24]. Similarly, provision of rapeseed meal, but not soybean meal, in the diet of sheep significantly lowered muscle cholesterol content [25]. These results suggest that some glucobrassicin derivatives (i.e. I3C or its acid condensation products) may be hypocholesterolemic.

The purposes of the present study were: (a) to synthesize and separate individual acid condensation products of I3C, (b) to assess the abilities of these compounds to inhibit ACAT activity, and (c) to investigate the physiological significance of this biochemical activity with respect to cholesterol homeostasis.

MATERIALS AND METHODS

Preparation of microsomes. Hepatic microsomes used for ACAT catalytic assays were derived from male CD-1 mice, purchased from Charles River Laboratories, Raleigh, NC. Animals (9 weeks old) were provided a 3% cholesterol (Sigma, St. Louis, MO)-enriched diet (Agway Prolab, Creedmoor, NC) for 1 week. Animals were killed by cervical dislocation; livers were excised quickly, and minced in ice-cold 1.15% KCl. Tissue was homogenized in 10 mM HEPES, pH 7.4, 1.0 mM EDTA, 10% glycerol using a Dounce tissue homogenizer. Homogenates were centrifuged at 10,000 *g* for 15 min, and the resulting supernatant was centrifuged at 100,000 *g* for 45 min. The 100,000 *g* pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4), 100 μM EDTA, 20% glycerol. Microsomal protein concentrations were measured according to Bradford [26] using commercially available reagent (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard.

ACAT assay. ACAT catalytic activity was determined according to Lichtenstein and Brecher [27] with the following modifications. ACAT activity was measured in a 400-μL incubation mixture containing 30 μg microsomal protein, 20 nmol fatty acid-free bovine serum albumin (Sigma), 0.1 M potassium phosphate buffer (pH 7.4), 1 mM EDTA, and 2 mM dithiothreitol (DTT). The mixture was preincubated for 6 min at 37° before initiating the reaction by the addition of 18 nmol [^{14}C]palmitoyl-CoA (Dupont, Boston, MA; 5.5 mCi/mmol). The reaction was allowed to proceed for 6 min at which time the reaction was terminated by the addition of 5 mL chloroform:methanol (2:1, v/v). Cholesteryl ester was extracted into the organic solvent, the solvent was evaporated under a stream of nitrogen, and the residue was spotted onto a TLC plate (Fisher Scientific Co., Pittsburgh, PA; silica gel G) with 60 μL of hexane:chloroform (8:1, v/v). The TLC plate was developed in a solvent system of hexane:ethyl ether:acetic acid (90:10:1, by vol.). Cholesteryl-[^{14}C]palmitate was visualized by autoradiography, and the film was used as a template to identify the position of cholesteryl-[^{14}C]palmitate on the TLC plate. The ester was cut from the plate and quantitated by liquid scintillation spectrometry. Identity of the cholesteryl-[^{14}C]palmitate was confirmed by its co-migration with an authentic standard (Sigma) and visualized with phosphomolybdic acid.

Synthesis and purification of the acid condensation products. The acid condensation products were synthesized according to Bradfield and Bjeldanes [28] with the following modifications. I3C (100 mg) was dissolved into 2 mL of dimethyl sulfoxide (DMSO), and the acid reaction was initiated by slowly adding the DMSO solution to 100 mL of 0.05 M HCl, pH 1.3. The solution was stirred constantly at room temperature for 80 min and, then extracted twice with 100 mL of methylene chloride. Next, the methylene chloride was filtered through a Millipore P5 filter packed with anhydrous magnesium sulfate to remove residual water. Methylene chloride was evaporated by rotary evaporation at 40°, and the residue was resuspended in 10 mL of acetonitrile.

Purification and separation of the individual acid condensation products were accomplished by HPLC using a preparative C_{18} reverse phase column and a mobile phase of 60% acetonitrile/40% water for 20 min at a flow rate of 4 mL/min. Products were detected by absorbance at 280 nm. Fractions containing specific acid condensation products were pooled, water was removed by phase separation with NaCl, and the acetonitrile was further dehydrated over anhydrous magnesium sulfate. Samples were stored in acetonitrile at −20°.

The inhibitory effects of the individual acid condensation products on microsomal ACAT activity were determined by adding the desired amount of acid condensation product to the ACAT assay tube and evaporating the acetonitrile with a stream of nitrogen prior to adding the other assay constituents. Inhibition curves were linearized by replotting the data using log concentrations, and IC_{50} values were calculated from the resulting linear regression equations.

Effect of orally administered I3C on cholesterol homeostasis. Mice were provided I3C in their diet for 1 week at an estimated dosage of 100 mg/kg/day. Feed was prepared by mixing I3C directly with ground feed (Agway Prolab: 22% protein, 5.0% fat, 5.0% fiber, 6.0% ash, 11% moisture), supplemented with 3% cholesterol, with a food processor. Cholesterol was supplemented to elevate low density lipoprotein/very low density lipoprotein (LDL/VLDL) cholesterol levels in the mice, which would

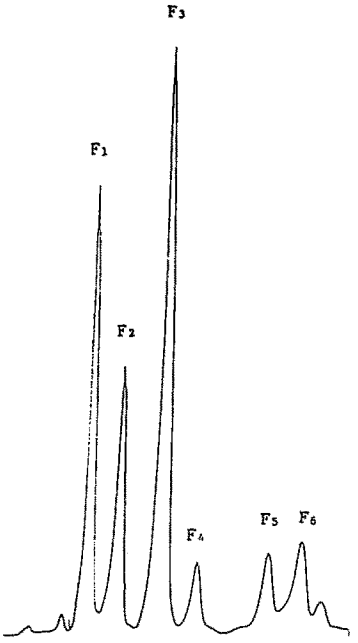

Fig. 1. HPLC chromatogram of the acid condensation products of I3C. Acid condensation products were synthesized *in vitro*, individual products were separated by HPLC using a C_{18} reverse phase column, and separated products (designated F_1 through F_6) were collected. Synthesis and purification techniques are described in detail in Materials and Methods.

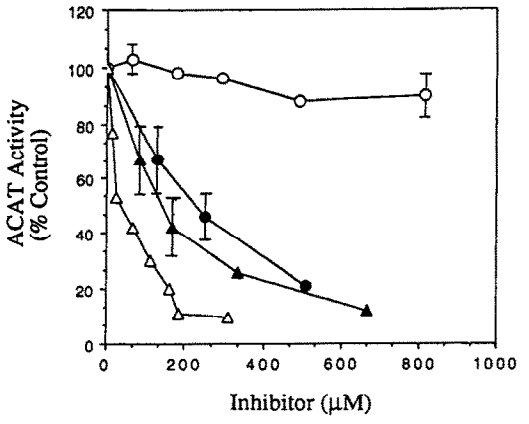

Fig. 2. Inhibition of microsomal ACAT activity by the acid condensation products of I3C. ACAT activity was measured [27] using hepatic microsomes derived from male CD-1 mice (details in Materials and Methods). Control specific activity was 416 ± 6 pmol/min/mg. Error bars represent the range of two assays. Key: (○) I3C; (△) CTI; (●) DIM; and (▲) BII.

enhance the ability to detect any hypocholesterolemic effects of I3C. Another group received only cholesterol-supplemented feed. Control animals were provided feed containing no I3C or cholesterol supplement. Each animal was provided 5.0 g of feed per day, and this quantity was entirely consumed daily. This dosage of I3C had no effect on mouse weight, liver weight, or general behavior and appearance. After treating animals for 7 days, mice were killed by cervical dislocation and blood was collected. Serum was prepared by allowing the blood to clot at 4° overnight. Samples were then centrifuged at 13,000 *g* for 10 min, and the serum was removed and stored at −20°. Serum total cholesterol levels were evaluated by the oxidase method [29], using commercially available reagents (Sigma). High density lipoprotein (HDL) cholesterol levels were determined by precipitating the LDL and VLDL with dextran sulfate and magnesium [30]. The HDL cholesterol was then measured using the same method as for total cholesterol. LDL/VLDL cholesterol was determined by subtracting the HDL cholesterol concentration from the total cholesterol concentration.

RESULTS

Synthesis and purification of the acid condensation products. The acid condensation products of I3C were synthesized *in vitro*, and six products (designated F_1 through F_6) were separated and purified by HPLC (Fig. 1). Products F_1 through F_6 eluted from the column at 6.7, 8.0, 9.8, 11.3, 14.3, and 15.7 min, respectively. The elution of products F_1, F_2, and F_3 corresponded to that of the dimeric, cyclic trimeric, and noncyclic trimeric derivatives 3,3′-diindolylmethane (DIM), 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole (CTI), and 2,3-bis[3-indolylmethyl]indole (BII), respectively [18]. The identity of F_1 was confirmed by its co-elution with DIM standard (provided by Dr. L. F. Bjeldanes, Department of Nutritional Studies, University of California, Berkeley, CA). F_4 and F_5 may be cyclic and noncyclic tetramers of I3C, respectively [31], while the elution time of F_6 suggests that it may be a cyclic pentameric derivative of I3C.

Inhibition of ACAT activity by the acid condensation products. The ability of I3C or its purified oligomeric derivatives to inhibit ACAT catalytic activity was determined next. The effects of the indoles on ACAT activity were measured using hepatic microsomes derived from mice provided a 3% cholesterol diet. Cholesterol administration significantly elevates hepatic microsomal ACAT activity [32], thus providing greater activity for the *in vitro* experiments. I3C did not inhibit ACAT activity at any of the concentrations tested (Fig. 2). However, several of the acid condensation products did inhibit ACAT activity (Fig. 2) with CTI being the most potent inhibitor (Table 1). These results indicate that, for the compounds examined, cyclic oligomers of I3C are more effective inhibitors of ACAT than the corresponding noncyclic derivatives and that the inhibitory potency of the cyclic acid condensation products decreases with increasing molecular mass.

Effect of I3C on cholesterol homeostasis. The inhibition of ACAT following the ingestion of I3C and its conversion to the acid condensation products in the stomach should impede the intestinal absorption and enhance the hepatic elimination of

Table 1. *In vitro* inhibition of microsomal ACAT activity by I3C and the purified acid condensation products of I3C

Inhibitor	Identity	IC_{50} ng/μL	IC_{50} μM
I3C	I3C	> 120	> 816
F_1	DIM	63	256
F_2	CTI	15	39
F_3	BII	50	133
F_4	—*	22	43*
F_5	—*	> 120	> 238*
F_6	—*	75	97*

ACAT activity was measured, and its inhibition by I3C and its products was determined as described in Materials and Methods.

* Molar concentrations were calculated based upon the assumption that F_4, F_5, and F_6 are cyclic tetrameric, noncyclic tetrameric, and cyclic pentameric derivatives of I3C, respectively.

cholesterol. Accordingly, orally administered I3C should mitigate the increase in serum LDL/VLDL cholesterol levels in mice provided a high cholesterol diet. Administration of a 3% cholesterol diet to mice for 1 week significantly ($P \leq 0.05$) elevated serum total cholesterol levels (Table 2). However, when cholesterol-fed mice were also provided 100 mg/kg I3C in their diet, the effect of dietary cholesterol on serum total cholesterol levels was attenuated by approximately 50% (Table 2). Thus, I3C significantly ($P \leq 0.05$) interfered with the accumulation of cholesterol in the blood of cholesterol-fed mice. Further, the hypocholesterolemic effect of I3C was entirely on LDL/VLDL cholesterol. Serum LDL/VLDL cholesterol levels were elevated 3-fold in cholesterol-fed mice but this effect was reduced significantly ($P \leq 0.05$) in I3C-treated, cholesterol-fed mice; serum HDL cholesterol levels were not affected by I3C (Table 2). These results further support the hypothesis that I3C can lower serum cholesterol levels by inhibiting ACAT.

DISCUSSION

Results from this study present a possible mechanism for the lowering of blood LDL/VLDL cholesterol levels through the consumption of glucobrassicin-rich vegetables. Processed vegetables, such as cabbage and broccoli, are rich in indole-3-carbinol [17]. We (Fig. 1) and others [18, 31, 33] have shown that under acid conditions, similar to those in the stomach, I3C is converted to various multimeric derivatives. Several of these derivatives have been detected in the stomach, small intestines, and liver of test animals following oral administration of I3C [18, 19]. Results from the present study demonstrated that several acid condensation products of I3C inhibit ACAT activity at micromolar concentrations (Table 1). Previous studies have demonstrated that up to 2.5% of the orally administered dose of I3C accumulates in the liver [34]. Assuming that the I3C accumulates in the liver primarily as dimeric or trimeric derivatives [18, 19], administration of the hypocholesterolemic dose of 100 mg/kg of I3C to mice would be expected to result in a liver concentration of I3C derivatives of approximately 100 μM. This concentration is within the ACAT-inhibitory concentration range for the acid condensation products of I3C, and suggests that, at this dosage, hepatic ACAT would be inhibited by these compounds.

The distribution of serum cholesterol between HDL and LDL/VLDL in mice on a high cholesterol diet was 53 and 47%, respectively (Table 2). Conversely, the distribution of cholesterol between HDL and LDL/VLDL in humans is approximately

Table 2. Effect of I3C on total and lipoprotein-specific serum cholesterol levels in cholesterol-fed mice

Cholesterol	Treatment	Serum cholesterol mg/dL	Serum cholesterol % of Control
Total	Control	112 ± 18	100
	3% Cholesterol	144 ± 21*	129
	3% Cholesterol + I3C	129 ± 7†	115
HDL	Control	89 ± 22	100
	3% Cholesterol	80 ± 14	90
	3% Cholesterol + I3C	80 ± 11	90
LDL/VLDL	Control	24 ± 15	100
	3% Cholesterol	72 ± 23*	300
	3% Cholesterol + I3C	52 ± 14†	217

Mice were provided 3% cholesterol in their diet or 3% cholesterol and I3C at an estimated daily dosage of 100 mg/kg for 1 week. Control mice received no supplements in their diet. Analyses were performed as described in Materials and Methods. Data are presented as means ± SD (N = 10–15).

* Significantly different from the control at $P \leq 0.05$ (Student's *t*-test).

† Significantly different from the 3% cholesterol treatment at $P \leq 0.05$ (Student's *t*-test).

35 and 65%, respectively [35]. Thus, the magnitude of effect of I3C on total cholesterol levels in humans would be appreciably greater than that observed in mice. For example, while the ~30% reduction in serum LDL/VLDL cholesterol described in Table 2 represented a 10% reduction in total cholesterol, this would be equivalent in humans to a lowering of total serum cholesterol from 240 to 193 mg/dL, a 20% reduction.

In addition to its role in the maintenance of cholesterol homeostasis via its activity in the intestines and liver, ACAT also has a direct role in arterial plaque formation. Atherogenesis consists of the accumulation of cholesteryl esters within the arterial wall [15, 16]. This process is characterized by the localization of macrophage cells within the arterial wall. Here, the macrophage cells accumulate cholesteryl esters derived from oxidized LDL via the scavenger receptor pathway [4]. In addition, free cholesterol within the macrophage plasma membrane becomes susceptible to ACAT-mediated esterification during the uptake of LDL by these cells [36]. The resulting cholesteryl ester-laden macrophage cells become foam cells, which constitute the major proportion of arterial plaque. The inhibition of ACAT, by I3C derivatives, within the macrophage cells would reduce the accumulation of cholesteryl esters within the cell and would facilitate the elimination of free cholesterol from these cells via HDL [16]. I3C has also been demonstrated to have antioxidant properties [34]. Since the oxidation of LDL is considered responsible for its uptake by macrophages resulting in foam cell formation [37], antioxidants, such as I3C, could protect against atherosclerosis by preventing foam cell formation [38]. Continued characterization of the hypocholesterolemic and possible anti-atherosclerotic properties of these indolic compounds may provide a rationale for conducting clinical evaluations of the hypocholesterolemic and anti-atherogenic properties of cruciferous vegetables and ultimately the establishment of dietary guidelines recommending the consumption of cruciferous vegetables to lower LDL cholesterol levels and reduce the risk of atherosclerosis.

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