Effect of Conjugated Linoleic Acids, Free and Esterified Linoleic Acids, and Trans-Vaccenic Acid on Rat Pulmonary 15-Lipoygenase-1 Activity

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Abstract

In the present study, the inhibition of rat pulmonary 15-Lipoygenase-1 (15-LOX-1) enzyme activity using Linoleic Acid (LA) as a substrate, was investigated by two of the major Conjugated Linoleic Acid (CLA) isomers, cis-9, trans-11 (c9,t11) and trans-10, cis-12 (t10,c12). Activity was measured by the production of 13(S)-Hydroxyoctadecadienoic acid [13(S)-HODE], an LA metabolite. Both CLA isomers c9,t11 and t10,c12 were found to inhibit 15-LOX-1 enzyme activity in a dose-dependent manner. 13(S)-HODE production from added 8µM CLA resulted in 33% and 57% reduction by c9,t11 and t10,c12-CLA, respectively. Interestingly from the addition of 80 µM trans-Vaccenic Acid (t-VA) also resulted in 51% reduction of 13(S)-HODE production. It was found that 80µM of c9,t11 or 100µM of t10,c12 or 100µM of t-VA were equivalent to 0.10µM Nordihydroguaiaretic Acid (NDGA) (LOX inhibitor) inhibition of the enzyme 15-LOX-1. The effect of esterified CLA on 15-LOX-1 activity was also investigated. Results showed that c9,t11-Conjugated Linoleic Acid Methyl Ester (c9,t11-CLAME) did not have any effect on 15-LOX-1 activity measured by the 13(S)-HODE production from LA. However using Linoleic Acid Methyl Ester (LAME) as a substrate, c9,t11-CLA was found to increase 13(S) HODE production in a dose dependent manner. The increases of 13(S)-HODE production were 21,63,146 and 350%, respectively compared to the control. If LAME was the substrate t10,c12-CLA and t-VA were found to be more effective as pulmonary 15-LOX-1 enzyme inhibitors than the c9,t11-CLA.

Introduction

Conjugated Linoleic Acid (CLA) is a mixture of positional and geometrical isomers of Linoleic Acid, cis-9, cis-12-octadecadienoic acid (LA). Of the CLA isomers, cis-9, trans-11 (c9,t11) and trans-10, cis-12 (t10,c12) are the most biologically active isomers [1,2]. The principal dietary form of CLA is c9,t11, however, the concentrations of c9,t11 and t10,c12 isomers in dairy products or meats from ruminants vary depending on the diet fed to animals [3,4]. CLA is produced by microbial conversion in the rumen from the biohydrogenation of polyunsaturated fatty acid, but also from the Δ9 desaturation of trans-Vaccenic Acid (t-VA), the predominant trans monoensaturated fatty acid of milk and animal tissue fat [5]. About 70% of c9,t11 present in milk fat is derived from the conversion of t-VA [6]. Trace of amounts of CLA may be also produced in vivo from free radical-mediated oxidation of LA [7], which consequently could form CLA isomers. However, it has been suggested that CLA in animal tissues may only be derived from dietary sources [8].

CLA isomers have been reported to have beneficial effects on cancer [9-12], atherosclerosis [13-15], immune function [16,17] inflammation [18-20] and body composition [21,22], and c9,t11 and t10,c12 isomers of CLA together might exhibit synergistic actions. Of the CLA isomers, t10,c12 was reported to be specially effective on body composition in mice. It should be noted that most of the studies were performed using the Free Fatty Acid (FFA) type of CLA, whereas most dietary CLA is in the Triglyceride (TG) form [23].

Dietary TG is degraded in vivo by lipase, and decomposed into 2-monoglyceride and two molecules of FFAs. Approximately 40 to 50% of TG is completely hydrolyzed to glycerol and FFAs prior to absorption, however, about 40 to 50% of fat is only partially hydrolyzed to mono- and diglycerides, and about 10% of the ingested lipid remains as TG or degraded to diglyceride [24].

Lipoxygenases (LOXs) are a class of non-heme iron dioxygenases found in plants, animals and microorganisms that insert molecular oxygen into free and/or esterified polyunsaturated fatty acids with...
regionally, and are designated 5-, 8-, 12-, and 15-LOX accordingly [24]. Two different 15-LOXs have been identified: 15-LOX-1 in the lungs and in skin tissue and its primary oxidation product is 13(S)-hydroxy-cis-9, trans-11-octadecadienoic acid (13(S)-HODE) [25]. 15-LOX-1 is predominantly expressed in reticulocytes, eosinophils, monocytes/macrophages, airway epithelial cells, atherosclerotic lesions, colorectal carcinomas, and prostate adenocarcinomas [26,27]. In numerous biological systems, 13(S)-HODE is involved in the regulation of cell proliferation and differentiation, and is suggested to be related to carcinogenesis [28]. Consequently, the inhibition of 13(S)-HODE production from LA, by the inhibition of 15-LOX-1, may lead to lower cell proliferation and tumor growth in biological systems. The purpose of this study was to (a) investigate the inhibition of c9,t11 and t10,c12-CLA on 15-LOX-1 activity when LA is used as a substrate, (b) determine whether c9,t11 methyl ester inhibits 15-LOX-1 activity using LA as substrate, and (c) determine the effect of c9,t11 and t10,c12-CLA on 15-LOX-1 activity using linoleic acid methyl ester as a substrate. Inhibition of c9,t11 and t10,c12-CLA, and t-VA on 15-LOX-1 activity were compared to Nordihydroguaiaretic Acid (NDGA) LOX inhibitor.

Materials and Methods

Materials

High purity Linoleic Acid (LA) and Linoleic Acid Methyl Ester (LAME), Nordihydroguaiaretic Acid (NDGA), and absolute ethanol were purchased from Sigma (St. Louis, MO). The purities of LA and LAME were both >99% by manufacturer’s certification. High purity cis-9, trans-11 and cis-10, cis-12-CLA, and cis-9, trans-11 CLA Methyl Ester (c9,t11-CLAME) were purchased from Matreya (Pleasant Gap, PA), and were also certified pure compounds >99%. Calcium chloride, tris (hydroxymethyl) aminomethane hydrochloride, and HPLC grade hexane, 2-propanol, acetonitrile, acetic acid and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ). 13(S)-Hydroxy-cis-9, trans-11-Octadecadienoic acid (13(S)-HODE) was purchased from Cayman Chemical (Ann Arbor, MI). Hydrochloric acid was purchased from Aldrich Chemical (Milwaukee, WI).

LA, LAME, c9,t11-CLA, t10,c12-CLA, and c9,t11-CLAME were stored as 200mM stock solutions in absolute ethanol at -20°C. The compound 13(S)-HODE was stored as 100mM stock solutions in absolute ethanol at -20°C. The LA, LAME and CLA isomers, CLAME, and 13(S)-HODE working solutions were prepared by diluting the stock solution with appropriate amounts of absolute ethanol before each experiment.

Lipoxygenase enzyme assay

Rat lung tissues and the preparation of 15-LOX-1 enzyme were the same as reported earlier [22,23]. The method of LOX assay was developed in this laboratory and described by Cho et al. [29]. Rat homogenates from lung (total protein concentration=400μg) were assayed for LOX activity by incubating with 18μM concentration of substrate (LA, LAME, c9,t11-CLA, t10,c12-CLA or c9,t11-CLAME) in ethanol (3% of final volume) at 37°C for 45 min in a total volume of 200μL containing 100mM Tris-HCl buffer (pH 7.2) and 2mM CaCl2. The reaction was stopped by the addition of 12μL of 0.2M hydrochloric acid. The major metabolite of each substrate, 13(S)-HODE, was extracted three times with 0.5mL of ethyl acetate. The ethyl acetate extracts were evaporated under nitrogen gas, and the residues were redissolved in 500μL of the mobile phase [hexane/2-propanol/acetonitrile/acetic acid (800:8:30:1, v/v/v)] used for the chromatography. The extracts were filtered on Millipore filters (Milford, MA, 0.45μm pore size) under very low pressure, and the samples and standards (50μL) were injected onto a normal phase silica High-Performance Liquid Chromatography (HPLC) column (Lichrosorb Si 60, 250×4mm I.D., 10μm; Alltech Associates Inc., Deerfield, IL) and the metabolites eluted at 0.9mL/min and measured by UV absorption at 235nm using a Waters 2847 dual λ absorbance detector (Milpore, Milford, MA). Peak areas were calculated by using Chromperfect chromatography software (Justice Laboratory Software, Palo Alto, CA).

Measurement of free radical type of oxidation of LA, LAME, c9,t11 and t10,c12-CLA, and c9,t11-CLAME

The content of 13(S)-HODE in free fatty acids and their methyl esters before and after incubation without the addition of 15-LOX-1 enzyme was measured by HPLC as described earlier.

5-LOX-1 enzyme induced oxidation of substrates measured by the net production of 13(S)-HODE

Enzyme induced oxidation for LA, LAME, c9,t11 and t10,c12-CLA, and c9,t11-CLAME was calculated after subtraction of the 13(S)-HODE produced by free radical type of oxidation, from the total 13(S)-HODE production, after incubation with the enzyme.

Inhibitory effect of c9,t11 or t10,c12-CLA on 15-LOX-1 activity using LA or LAME as substrate

Various concentrations (0, 0.1, 0.5 and 1μM of c9,t11 or t10,c12-CLA were incubated with substrate LA or LAME during 15-LOX-1 enzyme assay. Samples were treated as described in the above lipoxygenase enzyme assay.

Inhibitory effect of c9,t11-CLAME on 15-LOX-1 activity using LA or LAME as substrate

LA or LAME (18μM) was incubated with 0, 0.1, 0.5 and 1μM of c9,t11-CLAME during 15-LOX-1 enzyme assay. Samples were treated as described in the above lipoxygenase enzyme assay.

Inhibition of 15-LOX-1 activity by NDGA using LAME or c9,t11-CLAME as substrate

To test the activity of the isolated enzyme, various concentrations; 0.1, 0.5 and 1μM of NDGA were incubated during 15-LOX-1 enzyme assay when LAME or c9,t11-CLAME was used as a substrate.

Effect of LA and LAME on 15-LOX-1 activity using c9,t11-CLA as substrate

c9,t11-CLA was incubated with 0, 1, 2, 4 and 8μM LA or LAME. Samples were treated as described in the above lipoxygenase enzyme assay.

Inhibitory effect of trans-vaccenic acid on 15-LOX-1 activity using LA as substrate

T-VA (0, 1, 2, 4 and 8μM was incubated with substrate LA (18μM) and 15-LOX-1 enzyme activity was measured by the production of 13(S)-HODE.
**Statistical Analysis**

Results are presented as mean ± Standard Error of the Mean (SEM) of at least three replications of 13(S)-HODE production. Statistical analyses were conducted using SigmaStat statistical software package version 3.0 (Jandel Corp., San Rafael, CA). Statistical differences among treatments were tested by one-way Analysis of Variance (ANOVA) and the Student's t-test (p<0.05) where appropriate.

**Results and Discussion**

One of two known biologically active CLA isomers, c9,t11 has been associated with anticarcinogenic activity in various cancers, especially in mammary gland [9], skin [10], and colon [11,12]. The other isomer, t10,c12, recently has been shown to have a wide range of biological functions [15,24-31]. T-VA is a major trans monounsaturated fatty acid in dairy and animal tissue fat, and is converted to c9,t11 CLA by Δ9-desaturase reaction [5].

Enzymatic oxidation of LA is catalyzed either by lipoxygenase or cyclooxygenase depending on the tissue type [32]. The major 15-LOX-1 product from LA oxidation, 13(S)-HODE is involved in regulation of cell proliferation and differentiation in numerous biological systems [21-28]. This suggests that it may be directly or indirectly related to carcinogenesis. The inhibition of 13(S)-HODE production from LA by 15-LOX-1 may lead consequently to lower cell proliferation and tumor growth. In early studies, some trans fatty acids have been shown to be competitive inhibitors for soybean LOX-induced LA oxidation [33,34]. Since c9,t11 and t10,c12 CLA, and t-VA contain trans configuration, the possible inhibition of 15-LOX-1 activity and the consequent production of 13(S)-HODE was investigated in the present study. Since the major polyunsaturated fatty acid in the human diet is LA [35], inhibition of 15-LOX-1 activity is a matter of interest.

Isolated rat lung 15-LOX-1 was selected for studying the possible inhibition of the enzyme activity by CLA isomers since high level of 15-LOX-1 activity was reported in lung epithelial cells [36,37]. To verify the enzymatic oxidation of LA by isolated rat pulmonary 15-LOX-1, it was incubated with various concentrations of NDGA, a well established LOX inhibitor. It was found previously in this laboratory that NDGA inhibited enzymatic oxidation and 13(S)-HODE production in a dose-dependent manner. The reduction of 13(S)-HODE production from LA in the presence of 0.1, 0.5 and 1µM NDGA was 32, 52 and 71%, respectively [30].

The free radical type of oxidation was measured before and after 45 min incubation without the addition of 15-LOX-1 enzyme to LA, LAME, c9,t11-CLA, t10,c12-CLA, and c9,t11-CLAME. Contents of 13(S)-HODE was measured by peak area from HPLC analyses for LA, 9, 50, 719, LAME, 24, 741, c9,t11-CLA; 139, 734, t10,c12-CLA; 122, 108 and c9,t11-CLAME; 55, 549. Incubation had no further effect on 13(S)-HODE concentrations.

Comparison of the relative activities of rat pulmonary 15-LOX-1 is demonstrated in figure 1, using substrates as LA, LAME, c9,t11-CLA, c9,t11-CLAME and t10,c12-CLA. The relative levels of enzymatic oxidation were measured by 13(S)-HODE production. It can be seen that esterified LA and c9,t11-CLA are very limitedly used as substrate by 15-LOX-1. The relative activity of c9,t11-CLA as substrate is only 25% of the activity of LA and t10,c12-CLA is inactive. Compared to that of LA the activity of LAME and c9,t11-CLAME as substrates are only 7% and 1%, respectively. Again the nonenzymatic oxidations of LA, LAME, c9,t11-CLA, t10,c12-CLA, and c9,t11-CLAME were subtracted from the 13(S)-HODE production.

When rat pulmonary 15-LOX-1 enzyme homogenates were incubated with LA in the presence of c9,t11-, t10,c12-CLA and t-VA, both CLA isomers and t-VA inhibited the enzyme activity, and 13(S)-HODE production was suppressed in a dose-dependent manner (Figure 2). When 0.5, 1, 2, 4 and 8µM of c9,t11-CLA was incubated with LA; 8, 20, 22, 20 and 33% reduction of 13(S)-HODE production, respectively was observed. However, when t10,c12-CLA at 0.5, 1, 2, 4 and 8µM was incubated with LA; 26, 30, 43, 44 and 57% reduction of 13(S)-HODE production, respectively was observed. When t-VA at 0.5, 1, 2, 4 and 8µM was incubated with LA; 13, 31, 37, 50 and 51% reduction of 13(S)-HODE production, respectively was observed.

![Figure 1: Comparison of 15-LOX-1 activity measured by 13(S)-HODE production when LA, LAME, c9,t11-CLA, c9,t11-CLAME, and t10,c12-CLA used as a substrate under identical condition. The levels of 13(S)-HODE from non-enzymatic oxidation were subtracted from data. Values are expressed as mean ± SEM (n=6, collected from three independent experiments). Means with different letters are significantly different at p<0.01.](image1)

![Figure 2: Comparison of the dose-dependent inhibition of 15-LOX-1 enzyme activity using LA as a substrate, by c9,t11-CLA, t10,c12-CLA, or trans-Vaccenic Acid (t-VA) measured by the production of 13(S)-HODE. The levels of 13(S)-HODE from non-enzymatic oxidation were subtracted from data. Values are expressed as mean a SEM (n=8, collected from four independent experiments). Means with different letters are significantly different at p<0.05.](image2)
These results demonstrate that t10,c12-CLA and t-VA inhibit 15-LOX-1 activity more than does c9,t11-CLA. It was found that 8µM of c9,t11, 1µM of t10,c12-CLA or 1µM of t-VA were equivalent to 0.1µM NDGA in inhibition of the enzyme measured by 13(S)-HODE. Furthermore, it was found that 8µM t10,c12 and 8µM t-VA demonstrated the same amount of enzyme inhibition as did 0.5µM NDGA. Thus t10,c12 and t-VA are 8 times more effective in 15-LOX-1 inhibition than is c9,t11-CLA.

In food, t10,c12 isomer is present in minor quantities; commercial butter contains about 1.1% t10,c12 compared to 76.5% c9,t11 [38] and beef contains about 2.6% t10,c12 compared to 72.0% c9,t11 of total CLA [39]. Besides c9,t11 CLA, all the other isomers are creating only about 1% of the total CLA (t9,c9,t11,t13>8,c9,t10>t10,c12) [40]. Partially hydrogenated oils such as shortenings and margarines are the main sources of t10,c12 isomer. It was also reported that a minor isomer t9,t11 may be more potent inhibitor of tumorigenesis than t10,c12 [41]. It is interesting to note that the average intake of total CLA isomers in the US population is reported to be <500mg/day [42]. However, ruminant products contain about two times or more t-VA than c9,t11 CLA. Based on clinical studies, humans can convert t-VA to c9,t11 CLA. It was reported that ~20% of t-VA can be converted endogenously to c9,t11 CLA, therefore, the effective physiological dose of c9,t11 CLA is 1.4 times the c9,t11 CLA actual intake [43].

It should also be noted that most studies on CLA were reported using the unesterified FFA forms of CLA, whereas most dietary CLA is in the esterified TG form [22]. Dietary TG is degraded by lipase and decomposed into 2-monoglyceride and two molecules of FFA, and it was reported that c9,t11-CLA was esterified mainly in the sn-1 and/or sn-3 position (74%), and only 26% esterified in the sn-2 position. This indicates that the major part of c9,t11 isomer was released as FFA from the TG in the intestine and was absorbed as FFA from food such as butter and cheese [37]. Rumenic acid (c9,t11) was shown by Chardigny et al., [44] to be better absorbed, oxidized and incorporated into rat carcass from the external position (sn-1) than the internal position (sn-2) from dietary synthetic dioleoyl monourumeryl glycerol. Although some investigators have reported no significant differences between the physiological effects of free and esterified forms of CLA [45-48], our interest was to see the possible differences in two forms of CLA relating to 15-LOX-1 activity and, therefore, the production of 13(S)-HODE, which have been shown to relate to cell proliferation.

When various concentrations (0.5, 1, 2, 4 and 8µM) of c9,t11-CLAME were incubated with 15-LOX-1 using LA as substrate, 13(S)-HODE production was not affected. No significant differences were found in 13(S)-HODE production from LA with or without the incubation with c9,t11-CLAME.

The effect of CLA isomers on 15-LOX-1 activity was investigated if LAME is used as substrate (Figure 3). When LAME was incubated with 0.5, 1, 2, 4, 8µM c9,t11-CLA, the production of 13(S)-HODE was significantly increased (p<0.01). The increases of 13(S)-HODE production were 21, 63, 146, 264 and 350%, respectively compared to the control. However, when the same concentrations of t10,c12-CLA was incubated with 15-LOX-1 using LAME as substrate, no change in 13(S)-HODE production was observed. The results demonstrate that 15-LOX-1 can use LAME as a substrate but only in a very limited way. Small concentrations of c9,t11-CLA significantly increased the enzyme activity, however, t10,c12 did not increase the production of 13(S)-HODE from LAME.

Since 15-LOX-1 activity is only about 7% if LAME is used as substrate compared to LA, it is not surprising that the addition of c9,t11 increases enzyme activity, therefore, 13(S)-HODE production. Since t10,c12 was found previously not to be a substrate for 15-LOX-1 [23], addition of this CLA isomer did not change the limited enzyme activity of LAME.

To verify the enzymatic activity of the isolated pulmonary 15-LOX-1 when LAME was used as substrate, 0.1, 0.5 and 1µM NDGA, a LOX inhibitor, was used. Although the enzyme activity is relatively low when LAME is used as substrate, NDGA significantly inhibited the enzyme activity dose-dependently by 70, 91 and 93%, respectively (p<0.001) (Figure 4). When the inhibition of NDGA was measured with the same concentrations using c9,t11-CLAME as substrate, the enzyme activity was decreased by 20, 48 and 61%, respectively (p<0.05). The enzyme activity measured by the production of 13(S)-HODE from c9,t11-CLAME was about 20% of LAME without the addition of enzyme inhibitor NDGA. The enzyme activity for c9,t11-CLA was as mentioned earlier to be 25% of LA if used as substrate (Figure 1). However, when c9,t11-CLAME used as substrate, only 1.2% was the enzyme activity compared to LA. Nevertheless, this small activity seems to be enzymatic since it was significantly suppressed by 0.5µM NDGA.

Incremental additions of LA when c9,t11-CLA was used as a substrate very effectively increased the enzyme activity however the incremental additions of LAME was very different (Figure 5). Due to 0.5, 1, 2, 4 and 8µM of LA incubation with c9,t11 as substrate, 37, 60, 88, 109 and 137% increases in 13(S)-HODE productions were observed, respectively (p<0.05). However, the same amounts of LAME were incubated with the substrate, only 17, 34, 55, 79 and 105% increase of 13(S)-HODE productions were observed.
In conclusion, the present study demonstrates that both CLA isomers, c9,t11 and t10,c12, partial inhibitors for rat pulmonary 15-LOX-1 enzyme activities measured by the primary LA oxidation product 13(S)-HODE. We find that t10,c12-CLA and t-VA are more effective inhibitors of 15-LOX-1 than is the c9,t11-CLA isomer and that the methyl esters of LA and CLA have differing effects on 15-LOX-1 activity compared to the free acids.

In vitro studies of CLA have led to their consideration as therapeutic agents or as "Functional Foods" [49]. It is clear that dietary supplementation can increase CLA serum levels [50] but the therapeutic benefits thereof are less clear. A recent meta analysis of 8 studies with nine trials "did not support overall favorable effect of CLA supplementation BP regulation" [51]. Similarly Slojis [52] reported that a trial of CLA in overweight or obese individuals "did not support an antiatherosclerotic effect." It is tempting to speculate that the inhibitory effects described in the present study could contribute these therapeutic failures by reducing the endogenous production of 13(S) HODE from LA.

References