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# Conjugated Linoleic Acid, *cis*-9,*trans*-11, Is a Substrate for Pulmonary 15-Lipoxygenase-1 in Rat

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The objective of this study was to determine whether two of the major conjugated linoleic acid (CLA) isomers, *cis*-9,*trans*-11 (*c*9,*t*11) and *trans*-10,*cis*-12 (*t*10,*c*12), are possible substrates for pulmonary 15-lipoxygenase-1 (15-LOX-1) and, therefore, they are also involved in the production of 13(*S*)-hydroxyoctadecadienoic acid [13(*S*)-HODE] in biological systems. 13(*S*)-HODE, a major bioactive metabolite of linoleic acid, is an important intracellular signal agent and is involved in cell proliferation and differentiation in various biological systems. Nordihydroguaiaretic acid (NDGA), a known LOX inhibitor, was used as a control for measuring 15-LOX-1 enzyme activity. It was found that *c*9,*t*11-CLA was 25% as active as linoleic acid as a substrate for 15-LOX-1; however, *t*10,*c*12-CLA was not a substrate for 15-LOX-1 as measured by 13(*S*)-HODE production. The authenticity of the production of 13(*S*)-HODE from *c*9,*t*11 as a substrate was established by isolation and cochromatography with pure standard on HPLC using non-radioactive and [$^{14}$C]-*c*9,*t*11-CLA.

**KEYWORDS: Carcinogenesis; conjugated linoleic acid; 13-hydroxyoctadecadienoic acid; lipoxygenase; lung; rat**

## INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for positional and geometrical isomers deriving from linoleic acid, *cis*-9,*cis*-12-octadecadienoic acid (LA). Although a number of CLA isomers have been identified to date, *cis*-9,*trans*-11 (*c*9,*t*11) and *trans*-10,*cis*-12 (*t*10,*c*12) are the most biologically active isomers of CLA (*1*). Of the CLA isomers, *c*9,*t*11 was found to be the predominant form, comprising as much as 90% of the total (*2*). The above two isomers of CLA have been found to have a number of physiological activities, including anticarcinogenic (*3*−*7*), antiatherosclerotic (*8*), and antidiabetogenic (*9*) actions, although most research has focused on its anticarcinogenic activity. Almost all studies conducted thus far have used synthetic *c*9,*t*11-CLA. Consequently, the effect of individual isomers has not been fully investigated.

CLA may be produced in vivo from free radical-mediated oxidation of LA (*10*). Hydrogen abstraction by a free radical species and subsequent diene conjugation produces LA free radicals with a conjugated diene structure, which consequently form CLA isomers. However, CLA in animal tissues may also be derived from dietary sources. Dairy products in particular have been shown to contain significant amounts of CLA (*11*).

There is a large and growing body of evidence indicating that free radicals and radical-mediated oxidation processes play important roles in many pathological conditions including cancer (*12*).

Lipoxygenase (LOX) enzymes are a class of nonheme iron dioxygenases found in plants, animals, and microorganisms that catalyze the hydroperoxidation of polyunsaturated fatty acids. The LOX reaction proceeds with hydrogen abstraction, subsequently forming free radical intermediates and rearrangement of double bonds to form conjugated diene fatty acid hydroperoxides (*13*). The major oxidation product of the reaction of LA with LOX is 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid [13(*S*)-HPODE]. This hydroperoxide is rapidly converted further to 13(*S*)-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid [13(*S*)-HODE] by peroxidase. Because LA is the preferred substrate for 15-LOX-1, producing primarily 13(*S*)-HODE, LA is the major polyunsaturated fatty acid in the human diet (*14*). Therefore, 15-LOX-1 activity is an important factor in human metabolism.

The purpose of this study was to determine whether two of the major CLA isomers, *c*9,*t*11 and *t*10,*c*12, are possible substrates for 15-LOX-1 and, therefore, they are also involved in 13(*S*)-HODE production in biological systems.

## MATERIALS AND METHODS

**Materials.** Linoleic acid (LA) and nordihydroguaiaretic acid (NDGA) were purchased from Sigma (St. Louis, MO). CLA (*c*9,*t*11 and *t*10,*c*12) were purchased from Matreya (Pleasant Gap, PA). The purities of CLA isomers were both >99%. Tris(hydromethyl)aminomethane hydrochloride, calcium chloride, and HPLC grade hexane, 2-propanol, acetonitrile, acetic acid, and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ). 13(*S*)-Hydroxyoctadecadienoic acid [13(*S*)-HODE] was purchased from Cayman Chemical (Ann Arbor, MI). [1-$^{14}$C]-*c*9,*t*11-CLA (50−60 mCi/mmol) was purchased from Amersham (Arlington

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Heights, IL). Hydrochloric acid and 1,2-dimethylhydrazine dihydrochloride were purchased from Aldrich Chemical (Milwaukee, WI).

**Animals.** Rat lung tissues were obtained from a separate study for the isolation of enzyme. The animals were treated as follows: 15 weanling Wistar (35−50 g) male rats (Harlan Sprague−Dawley, Inc., Indianapolis, IN) were fed a modified AIN 93-G diet containing 20% corn oil for 11 weeks. The animals were given two treatments with the colonic carcinogen 1,2-dimethylhydrazine dihydrochloride at a dose of 15 mg/kg of body weight by gavage. The average body weight of rats was 573.4 ± 13.3 g at the end of the feeding period. The animals were anesthetized and asphyxiated with $CO_2$, and the lung tissues were collected, quickly frozen with dry ice, and kept at −70 °C until analyzed for 15-LOX-1 activity.

**Lipoxygenase Enzyme Preparation.** The lung tissues from rats were homogenized in 1:3 (w/v) ice-cold 100 mM Tris-HCl buffer (pH 7.2), using a Polytron homogenizer (Brinkmann Instrument, Westbury, NY) at 4 °C. The homogenate was centrifuged at 9000*g* at 4 °C for 20 min. The supernatant fraction was collected and recentrifuged at 100000*g* for 1 h. The resulting supernatant fraction was collected and used for the LOX enzyme assay. Protein concentration was determined according to the Bradford protein assay (*15*) with bovine serum albumin used as standard.

**Lipoxygenase Enzyme Assay.** The LOX assay procedure was developed with slight modification in this laboratory as described by Cho et al. (*16*). Rat lung enzyme homogenates (total protein concentration = 400 μg) were incubated with 18 μM concentration of substrate (LA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA) in ethanol (3% of final volume) at 37 °C for 45 min, in a total volume of 200 μL containing 100 mM Tris-HCl buffer (pH 7.2) and 2 mM $CaCl_2$. The reaction was terminated by the addition of 12 μL of 0.2 M HCl. The major metabolite of each substrate, 13(*S*)-HODE, was extracted three times with 0.5 mL of ethyl acetate. The ethyl acetate extracts were evaporated under nitrogen gas, and the residues were redissolved in 500 μL of the mobile phase [hexane/2-propanol/acetonitrile/acetic acid (800:8:30:1, v/v)] used for the chromatography. Samples and standards (50 μL) were injected onto a normal phase silica high-performance liquid chromatography (HPLC) column (Lichrosorb Si 60, 250 × 4 mm i.d., 10 μm; Alltech Associates Inc., Deerfield, IL), and the metabolites were measured by UV absorption at 235 nm using a Waters 2847 dual λ absorbance detector (Millipore, Milford, MA) and peak areas were calculated using Chromperfect software (Justice Laboratory, Palo Alto, CA).

**Inhibition of 15-LOX-1 Activity.** Various concentrations (0.1, 0.5, and 1 μM) of NDGA were incubated when substrate LA or *c*9,*t*11 was used. Samples were treated as described in the above lipoxygenase enzyme assay.

**Verification of *c*9,*t*11-CLA as Substrate for 15-LOX-1.** Rat lung homogenate was incubated with a mixture of 18 μM non-radioactive *c*9,*t*11-CLA and [1-$^{14}$C]-*c*9,*t*11-CLA (2 μCi) in ethanol. Samples were treated as described in the above lipoxygenase enzyme assay. The HPLC fractions corresponding to [$^{14}$C]-13(*S*)- and [$^{14}$C]-9(*S*)-HODE were collected. Radioactivity was determined in a liquid scintillation counter (Beckman LS 5000TD, Fullerton, CA), and cochromatography with authentic material was conducted.

**Statistical Analysis.** Values of 13(*S*)-HODE production were presented as mean ± standard error of the mean (SEM) of at least five replications. Statistical analyses were conducted using SigmaStat version 3.0 (Jandel Corp., San Rafael, CA). Differences among treatments were determined using one-way analysis of variance (ANOVA) and Student's *t* test ($p < 0.05$).

## RESULTS

**Dose-Dependent Inhibition of NDGA on 15-LOX-1 Activity Using LA as Substrate.** Rat pulmonary 15-LOX-1 activity was measured by 13(*S*)-HODE production from LA substrate using HPLC. The total area of 13(*S*)-HODE was 17 084 785 from which 9 050 719 (~53% of total) was subtracted as a background due to oxidation in the absence of the enzyme (**Figure 1**). The background production of 13(*S*)-HODE (9 050 719) due to nonenzymatic oxidation was determined using assay conditions and HPLC measurement identically as described in the enzyme assay above, but without the addition of 15-LOX-1 enzyme. In **Figures 1** and **6**, only the enzymatic oxidation of 13(*S*)-HODE is illustrated.

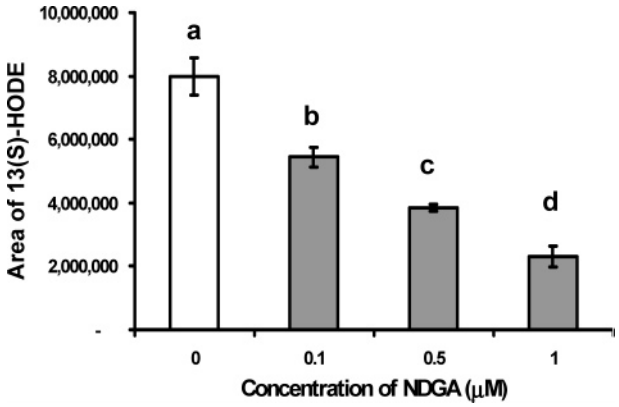


**Figure 1.** Dose-dependent inhibition of NDGA on rat pulmonary 15-LOX-1 activity, measured by HPLC, of 13(*S*)-HODE production from LA as substrate. Levels of 13(*S*)-HODE represent activity of 15-LOX-1. The production of 13(*S*)-HODE due to free radical type of oxidation was subtracted from data. Values are expressed as mean ± SEM ($n = 6$, collected from three independent experiments). Means with different letters are significantly different at $P < 0.005$. NDGA, nordihydroguaiaretic acid.

The effect of NDGA, a LOX inhibitor, is shown by the suppression of 15-LOX-1 enzyme-induced oxidation and measured by the production of 13(*S*)-HODE. A significant dose-dependent inhibition of LA oxidation by the enzyme was observed when 0.1, 0.5, or 1 μM NDGA was added to the reaction mixture, and 13(*S*)-HODE production was decreased by 32, 52, and 71%, respectively.

**Demonstration of 15-LOX-1 Activity Measured by 13(*S*)-HODE Production Using *c*9,*t*11-CLA as Substrate.** 13(*S*)-HODE formation and separation by HPLC were demonstrated when *c*9,*t*11-CLA was used as substrate for 15-LOX-1 instead of LA.

Clear separation of 13(*S*)-HODE (retention time = 35 min) is demonstrated from other eluting compounds in **Figure 2**. After incubation of CLA with 15-LOX-1, total oxidation products were 29.5%. Of the total oxidation products, 61.4% was enzymatic and 38.6% was nonenzymatic. Of the total enzymatic oxidation products, 5.2% was 13(*S*)-HODE.

**Verification of *c*9,*t*11-CLA as Substrate for 15-LOX-1 by Comparison of Retention Times of 13(*S*)-HODE.** Verification that *c*9,*t*11-CLA is a substrate for 15-LOX-1 was made by the identification of 13(*S*)-HODE production by the enzyme. Identification was achieved by comparison with (a) the non-radioactive 13(*S*)-HODE retention time (**Figure 2**), (b) the elution profile of [$^{14}$C]-13(*S*)-HODE (**Figure 3**), and (c) the retention time of 13(*S*)-HODE standard (**Figure 4A**). The isolated non-radioactive 13(*S*)-HODE, [$^{14}$C]-13(*S*)-HODE, and the 13(*S*)-HODE standard eluted at the same retention time, 35 min.

**Verification of *c*9,*t*11-CLA as Substrate for 15-LOX-1 by Cochromatography.** The isolated [$^{14}$C]-13(*S*)-HODE peak, which was mixed with pure 13(*S*)-HODE standard (**Figure 4B**), eluted together at 35 min. The mobile phase used contains 95% hexane, and the lack of absorbance of hexane at 235 nm at a retention time of 35 min is demonstrated in **Figure 4**.

For further confirmation, the [$^{14}$C]-13(*S*)-HODE peak, which eluted at 35 min (**Figure 4B**), was collected and rechromatographed in the same system. The repurified [$^{14}$C]-13(*S*)-HODE (**Figure 5**) showed the same elution time at 35 min as the pure

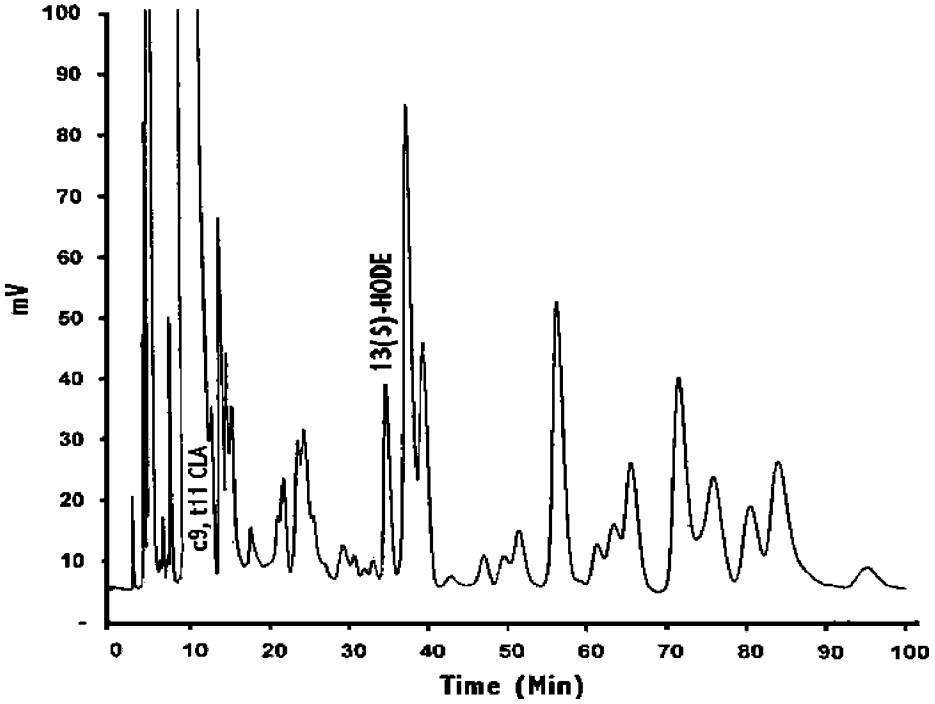


**Figure 2.** 13(*S*)-HODE production and separation by HPLC in the presence of 15-LOX-1 using *c*9,*t*11-CLA as substrate. Products were produced by incubation of isolated rat lung homogenate (400 $\mu$g of protein concentration) and 18 $\mu$M *c*9,*t*11-CLA as substrate for 45 min at 37 °C. Separation conditions: Lichrosorb Si 60 (250 × 4 mm i.d., 10 $\mu$m) normal phase column; isocratic elution with hexane/2-propanol/acetonitrile/acetic acid (800:8:30:1, v/v); flow rate, 0.9 mL/min; detector wavelength, 235 nm; detector response in mV; sample injection, 50 $\mu$L.

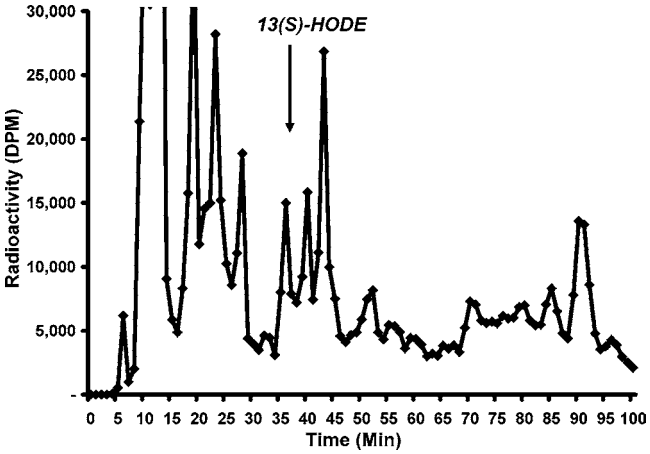


**Figure 3.** HPLC separation of [$^{14}$C]-13(*S*)-HODE from unreacted [$^{14}$C]-*c*9,*t*11-CLA and other compounds. Radioactive products were produced identically as described in **Figure 2** except 2 $\mu$Ci of [1-$^{14}$C]-*c*9,*t*11-CLA was added to the reaction mixture. Separation conditions are given in the caption of **Figure 2**, except the injection volume was 250 $\mu$L, and 0.9 mL fractions were collected for 100 min.

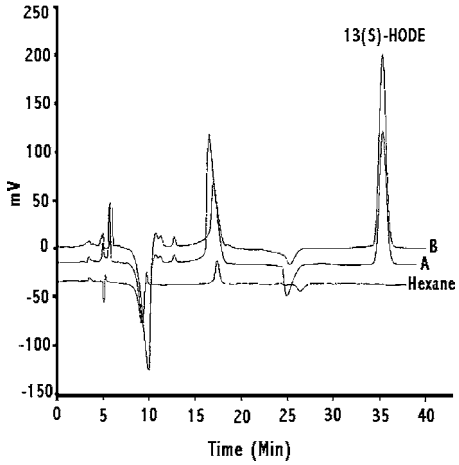


**Figure 4.** Cochromatography of 13(*S*)-HODE and [$^{14}$C]-13(*S*)-HODE on a normal phase HPLC column: (**A**) pure 13(*S*)-HODE standard; (**B**) pure 13(*S*)-HODE standard mixed with the isolated [$^{14}$C]-13(*S*)-HODE (1:1 ratio) fractions (35–38) collected by chromatography of compounds described in **Figure 3**; (**Hexane**) baseline chromatography of pure hexane, which is 95% of the mobile phase. Separation conditions are given in **Figure 2** except that the injection volume was 250 $\mu$L.

13(*S*)-HODE standard (**Figure 4A**). The recovery of 13(*S*)-HODE peak area from cochromatography detected by UV absorption was 96.9% (**Figure 4B**). After two repeated purifications by HPLC, the radioactivity of the isolated [$^{14}$C]-13(*S*)-HODE was 70.3% (**Figure 5**).

**Demonstration of 15-LOX-1 Activity Measured by 13(*S*)-HODE Production Using *t*10,*c*12-CLA as Substrate.** No 13(*S*)-HODE was produced from *t*10,*c*12-CLA when used as a substrate for rat pulmonary 15-LOX-1; therefore, it is concluded that *t*10,*c*12 is not a substrate for 15-LOX-1.

**Dose-Dependent Inhibition of 15-LOX-1 to NDGA Measured by 13(*S*)-HODE Production Using *c*9,*t*11-CLA as Substrate.** A potent dose-dependent inhibition of 15-LOX-1 was found when 0.1, 0.5, or 1 $\mu$M NDGA was incubated with the enzyme and *c*9,*t*11 used as a substrate. The inhibition of 13(*S*)-HODE production was 90, 96, and 96%, respectively (**Figure 6**).

## DISCUSSION

Conjugated linoleic acid, especially the *c*9,*t*11 isomer, has been associated with anticarcinogenic activity in cancers of the stomach (*3*), skin (*4*), colon (*6*), and mammary gland (*5*, *7*). This isomer can be found in dairy and meat products due to its production by microorganisms in ruminants, providing a dietary

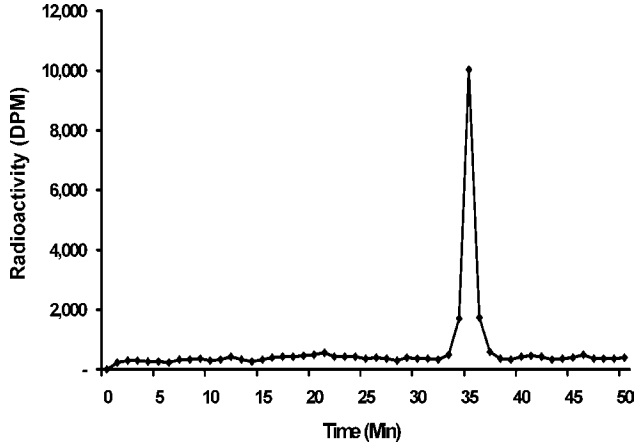


**Figure 5.** Rechromatography of collected peak (fractions 35–38) shown in **Figure 4B** for verification of the unity of the isolated [$^{14}$C]-13(**S**)-HODE and pure 13(*S*)-HODE standard on the normal phase HPLC column described in **Figure 2**, except that the injection volume was 250 $\mu$L.

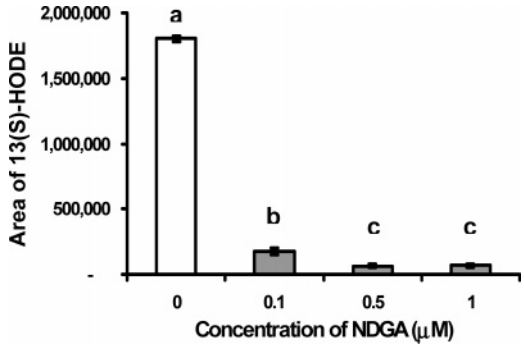


**Figure 6.** Dose-dependent inhibition of NDGA on 15-LOX-1 activity measured by 13(*S*)-HODE production using *c*9,*t*11-CLA as substrate. Levels of 13(*S*)-HODE represent oxidation by 15-LOX-1. The levels of 13(*S*)-HODE from nonenzymatic oxidation were subtracted from data. Values are expressed as mean ± SEM ($n = 6$, duplicate determinations in three independent experiments). Means with different letters are significantly different at $P < 0.005$.

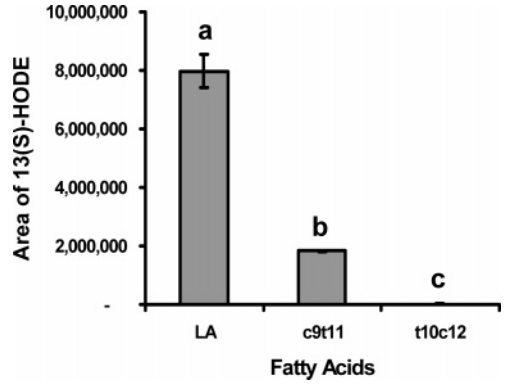


**Figure 7.** Comparison of 13(*S*)-HODE production from LA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA by 15-LOX-1. Levels of 13(*S*)-HODE represent oxidation by 15-LOX-1. Levels of 13(*S*)-HODE from nonenzymatic oxidation were subtracted from data. Values are expressed as mean ± SEM ($n = 6$, collected from three independent experiments). Means with different letters are significantly different at $P < 0.001$.

source for humans. The other most investigated isomer of CLA, *t*10,*c*12, has recently been shown to have a wide range of biological functions (*1*), but its amount in dairy products and meat is minimal compared to the *c*9,*t*11 isomer.

The oxidation of LA can be induced nonenzymatically by free radicals or catalyzed enzymatically by either lipoxygenase or cyclooxygenase depending on the tissue type (*17*). Oxidation products of LA are involved in a number of aspects of cellular regulation, both in physiological and in pathological processes. The major 15-LOX-1 product derived from LA oxidation is 13-(*S*)-HODE, which is involved in the regulation of cell proliferation and differentiation in numerous biological systems (*18*, *19*) and, therefore, is suggested to be related to carcinogenesis.

In the present study, it was investigated whether CLA isomers could be possible substrates for 15-LOX-1 and involved in the production of 13(*S*)-HODE related to increased cell proliferation. When *c*9,*t*11-CLA was used as a substrate for rat pulmonary 15-LOX-1, 13(*S*)-HODE was produced, indicating enzymatic oxidation of this CLA isomer.

To verify the enzymatic activity of isolated rat pulmonary 15-LOX-1 using LA as a substrate, it was incubated with various concentrations of NDGA, and 13(*S*)-HODE production was measured. NDGA, a well-established LOX inhibitor, reduces ferric ion to the inactive ferrous form, and the catechol substituent in NDGA is oxidized to the semiquinone (*20*). NDGA inhibited 13(*S*)-HODE production from LA in a dose-dependent manner, showing enzymatic oxidation. The reduction of 13(*S*)-HODE produced from LA in the presence of 1 $\mu$M NDGA was ~71%. Nonenzymatic oxidation of LA due to incubation in the reaction mixture was subtracted from the total 13(*S*)-HODE production. The net 13(*S*)-HODE production due to enzymatic activity is shown in **Figure 1**.

The separation and isolation of 13(*S*)-HODE were successfully achieved by HPLC, as illustrated in **Figure 2**. For the verification of 13(*S*)-HODE production, using *c*9,*t*11-CLA as substrate for 15-LOX-1, cochromatography of 13(*S*)-HODE was carried out with a mixture of (a) 13(*S*)-HODE standard and (b) isolated and purified [$^{14}$C]-13(*S*)-HODE (from previous reactions of lung 15-LOX-1 with a mixture of $^{14}$C-labeled and non-radioactive *c*9,*t*11-CLA as substrate) (**Figure 4B**). The isolated [$^{14}$C]-13(*S*)-HODE peak mixed with 13(*S*)-HODE standard eluted together at 35 min. After repurification by HPLC, the recovery of the isolated 13(*S*)-HODE from enzymatic oxidation of *c*9,*t*11-CLA was 96.9% by UV and 70.3% by $^{14}$C radioisotope analysis (**Figure 5**).

To further verify the enzymatic oxidation of *c*9,*t*11-CLA by the isolated rat pulmonary 15-LOX-1, the enzyme was inhibited with various concentrations of NDGA; 1 $\mu$M NDGA reduced enzyme activity by 96% as measured by the diminished formation of 13(*S*)-HODE (**Figure 6**). In **Figure 7**, comparison of production of 13(*S*)-HODE from LA, *c*9,*t*11-CLA, and *t*10,*c*12-CLA by 15-LOX-1 is demonstrated.

From the present experiment, it is concluded that *c*9,*t*11-CLA can be a substrate in the production of 13(*S*)-HODE by 15-LOX-1. However, it was found that *t*10,*c*12-CLA was not a substrate for 15-LOX-1 because there was no enzymatic oxidation of this CLA isomer and, therefore, no 13(*S*)-HODE production occurred.

In conclusion, the present study shows that *c*9,*t*11-CLA has ~25% the activity as a substrate for 15-LOX-1 compared to LA measured by the enzymatic production of 13(*S*)-HODE, but the *t*10,*c*12-CLA isomer is not a substrate for the enzyme.

## ABBREVIATIONS USED

CLA, conjugated linoleic acid; LA, linoleic acid; *c*9,*t*11-CLA, *cis*-9,*trans*-11-CLA; *t*10,*c*12-CLA, *trans*-10,*cis*-12-CLA; HODE, hydroxy-*cis*-9,*trans*-11-octadecadienoic acid; NDGA, nordihydroguaiaretic acid; LOX, lipoxygenase; HPODE, hydroperoxy-

*cis*-9,*trans*-11-octadecadienoic acid; HPLC, high-performance liquid chromatography.

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