

Cannabidiol-2',6'-dimethyl Ether as an Effective Protector of 15-Lipoxygenase-Mediated Low-Density Lipoprotein Oxidation *in Vitro*

Shuso TAKEDA,^a Akari HIRAYAMA,^a Shino URATA,^a Nobutaka MANO,^a Keiko FUKAGAWA,^a Midori IMAMURA,^a Ayumi IRII,^a Satomi KITAJIMA,^a Tomoko MASUYAMA,^a Mai NOMIYAMA,^a Sachiko TATEI,^a Saari TOMITA,^a Taichi KUDO,^a Momoko NOGUCHI,^a Yasuhiro YAMAGUCHI,^a Yoshiko OKAMOTO,^a Toshiaki AMAMOTO,^b Yoshifumi FUKUNISHI,^c Kazuhito WATANABE,^d Curtis John OMIECINSKI,^e and Hironori ARAMAKI^{*,a}

^aDepartment of Molecular Biology, Daiichi University of Pharmacy; 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan; ^bNEUES Corporation; Yaesu Center Building 3F, 1-6-6 Yaesu, Chuo-ku, Tokyo 103-0028, Japan; ^cBiomedical Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST); 2-3-26 Aomi, Koto-ku, Tokyo 135-0064, Japan; ^dDepartment of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University; Ho-3 Kanagawa-machi, Kanazawa 920-1181, Japan; and ^eCenter for Molecular Toxicology and Carcinogenesis; 101 Life Sciences Building, Pennsylvania State University, University Park, PA 16802, United States. Received February 17, 2011; accepted May 6, 2011; published online May 23, 2011

15-Lipoxygenase (15-LOX) is one of the key enzymes responsible for the formation of oxidized low-density lipoprotein (ox-LDL), a major causal factor for atherosclerosis. Both enzymatic (15-LOX) and non-enzymatic (Cu^{2+}) mechanisms have been proposed for the production of ox-LDL. We have recently reported that cannabidiol-2',6'-dimethyl ether (CBDD) is a selective and potent inhibitor of 15-LOX-catalyzed linoleic acid oxygenation (Takeda *et al.*, *Drug Metab. Dispos.*, 37, 1733–1737 (2009)). In the LDL, linoleic acid is present as cholesteryl linoleate, the major fatty acid esterified to cholesterol, and is susceptible to oxidative modification by 15-LOX or Cu^{2+} . In this investigation, we examined the efficacy of CBDD on i) 15-LOX-catalyzed oxygenation of cholesteryl linoleate, and ii) ox-LDL formation catalyzed by 15-LOX *versus* Cu^{2+} -mediated non-enzymatic generation of this important mediator. The results obtained demonstrate that CBDD is a potent and selective inhibitor of ox-LDL formation generated by the 15-LOX pathway. These studies establish CBDD as both an important experimental tool for characterizing 15-LOX-mediated ox-LDL formation, and as a potentially useful therapeutic agent for treatment of atherosclerosis.

Key words cannabidiol-2',6'-dimethyl ether; 15-lipoxygenase; low-density lipoprotein; atherosclerosis; cannabinoid

15-Lipoxygenase (15-LOX) belongs to the structurally and functionally related family of non-heme iron dioxygenases. Three major LOX isoforms have been identified, 5-, 12-, and 15-LOX.¹⁾ In particular, 15-LOX has emerged as an attractive target for therapeutic intervention, as this pathway is suggested to play an important role in the atherosclerosis disease process.^{2,3)} Although atherosclerosis is a multifactorial syndrome that involves chronic inflammation at every stage, from initiation to progression and eventually plaque rupture, oxidized low-density lipoprotein (ox-LDL) is recognized as a key mediator in the disease development.⁴⁾ Following its generation, ox-LDL is internalized by macrophages through scavenger receptors such as CD36, a type B scavenger receptor, leading to the process that in turn stimulates the cyclic progression of atherosclerotic disease.⁴⁾ Enzymatic contributors to the formation of ox-LDL include 15-LOX, myeloperoxidase, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.⁵⁾ Among these, results from DNA microarray, transgenic and knockout mouse studies suggest an important role of 15-LOX in the disease etiology.^{5–7)} However, ox-LDL may also be formed non-enzymatically, for example through Cu^{2+} -mediated oxidation,⁸⁾ and therefore the specific contributory roles of enzymatic *versus* non-enzymatic of ox-LDL generation remains to be determined. Although it is reported that oxidation of LDL by 15-LOX can be processed *via* direct reaction with phospholipids,^{9,10)} 15-LOX directly oxygenates both polyunsaturated fatty acids (PUFAs) and cholesteryl esters formed between the carboxyl group ($-\text{COOH}$) of PUFAs and the hydroxyl

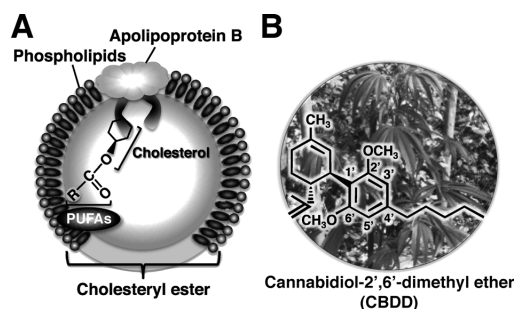


Fig. 1. Structures of the LDL Particle and CBDD

(A) Polyunsaturated fatty acids (PUFAs) are contained as cholesteryl esters in the LDL. 'R' in the Figure denotes a hydrocarbon chain of a respective fatty acid. (B) Structure of CBDD is shown. The numbered ring positions in CBDD correspond to the monoterpene nomenclature.

group ($-\text{OH}$) of cholesterol (see Fig. 1A).^{2,11,12)} Among the cholesteryl esters in LDL, cholesteryl linoleate is the most abundant substrate for fatty acid oxidation and represents the major oxidizable lipid.^{9,11,13)} Further, LOX enzymes can generate the formation of conjugated dienes and lipid hydroperoxides that may be obligatory intermediates in LDL oxidative modification.^{9,14)} To more clearly dissect the contributing roles of 15-LOX *versus* non-enzymatic pathways in LDL oxidation, in this study we utilized a highly potent and selective inhibitor of 15-LOX-catalyzed linoleic acid oxygenation, cannabidiol-2',6'-dimethyl ether (CBDD), recently identified by our laboratory.¹⁵⁾ CBDD exhibits an IC_{50} potency of $0.28 \mu\text{M}$ and selectivity indicated by a 5-LOX/15-LOX ratio

* To whom correspondence should be addressed. e-mail: haramaki@daiichi-cps.ac.jp

of IC_{50} values >700 .¹⁵⁾ CBDD was synthesized from cannabidiol (CBD) which is a major component of leaf of the fibrous plant, *Cannabis sativa*, via methylation of the resorcinolic OH groups.¹⁵⁾ Specifically, we hypothesized that: i) CBDD may inhibit conjugated diene formation in cholesteryl esters, especially that of cholesteryl linoleate, and ii) inhibit the enzymatic formation of ox-LDL catalyzed by 15-LOX, but not by the non-enzymatic pathway of Cu^{2+} -mediated oxidation.

The results presented demonstrate that CBDD effectively abrogates the enzymatic pathway of ox-LDL formation, likely by inhibiting the 15-LOX mediated oxidative modification of cholesteryl linoleate, but not the Cu^{2+} -mediated oxidation pathway of ox-LDL generation.

MATERIALS AND METHODS

Reagents Δ^9 -Tetrahydrocannabinol (Δ^9 -THC) was isolated and purified from the cannabis leaves according to the methods described elsewhere.^{15,16)} CBD and CBDD were purchased from Tocris Bioscience (Ellisville, MO, U.S.A.) and Cayman Chemical Co. (Ann Arbor, MI, U.S.A.), respectively. Purity of Δ^9 -THC was $>95\%$, as assessed by gas chromatography.^{15,16)} Arachidonic acid, linoleic acid, and nordihydroguaiaretic acid (NDGA) was purchased from Cayman Chemical Co. Human plasma LDL (purity $>95\%$) (lot nos. D00067046 and D00096308) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Cholesteryl linoleate was purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Enzyme Sources Measurements of the 5-LOX and 15-LOX activities were conducted using a commercially available LOX inhibitor screening assay kit (Cayman Chemical Co.). 5-LOX (lot No. 0400028—1) and 15-LOX (lot No. 0406588—4) enzymes were purchased from Cayman Chemical Co. All inhibitors added to the reaction system were prepared immediately prior to use. Following the enzymatic reactions, the resulting hydroperoxides were treated with chromogen to develop the reaction and then absorbance intensities were determined spectrophotometrically at 490 nm with a Molecular Devices E^{max} Precision Microplate Reader (Molecular Devices, Inc., Philadelphia, PA, U.S.A.). No colorimetric change was observed in control incubations performed in the absence of enzymes or with heat-denatured enzymes when added with chemical inhibitors and chromogen. The concentration ranges of compounds used in this study were determined empirically based on their solubilities and levels demonstrated not to interfere with chromogen detection. Each assay was performed in triplicate.

Assay for Formation of Conjugated Dienes 15-LOX and PUFAs (arachidonic acid or linoleic acid) or cholesteryl linoleate were incubated in the presence or absence of CBDD or CBD for various times in spectrophotometric cuvettes (1.0 cm light path). Incubations were performed in 100 mM borate buffer, pH 9.0 at room temperature.^{17,18)} Absorbance at 234 nm was periodically recorded after addition of 15-LOX. Results are expressed as an increase in absorbance from the zero time reaction mixture.

Ox-LDL Preparation and Agarose Gel Electrophoresis Immediately prior to the experiments, extensive dialysis was performed to remove ethylenediaminetetraacetic acid (EDTA)

added during the isolation of human LDL. After dialysis, LDL was used either immediately or subsequent to storage for <48 h at 4°C in the dark and under argon. Protein contents were determined by the Bradford protein assay reagent (Bio-Rad Laboratories Inc., Richmond, CA, U.S.A.) or Precision Red Advanced Protein Assay Reagent #2 (Cytoskeleton Inc., Denver, CO, U.S.A.). Oxidation of LDL into ox-LDL, mediated by copper (Cu^{2+} , $CuSO_4$)-induced non-enzymatic oxidation or 15-LOX-induced enzymatic oxidation, was determined at 37°C for 5 h, in the presence or absence of CBDD. Subsequent to separation by agarose gel electrophoresis, LDL was stained with Coomassie Brilliant Blue (CBB) G250 (Bio-Safe™ Coomassie Stain, Bio-Rad Laboratories Inc.). No observable formation of ox-LDL was detected for 2 h incubation. Further details of the experiments are described in the legend to Fig. 5 and the cited literature.⁵⁾

Western Blot Analysis of ApoB After incubation with 15-LOX or Cu^{2+} , resulting ox-LDLs were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (4% acrylamide gel), and then Western blot analysis was performed according to the methods previously.¹⁹⁾ An antibody to human apolipoprotein B (predicted molecular weight of 516 kDa) was used (Abcam, Cambridge, MA, U.S.A.).

Data Analysis The IC_{50} value was determined from the curves plotting enzymatic activity *versus* inhibitor concentrations. The details of the calculations were described previously.^{15,16,20)} All statistical analyses were performed by Scheffé's *F* test, a *post-hoc* test for analyzing results of analysis of variance (ANOVA) data. The calculations were performed using Statview5.0J software (SAS Institute Inc., Cary, NC, U.S.A.). Differences were considered significant at *p* values <0.05 .

RESULTS AND DISCUSSION

CBDD Inhibits 15-LOX-Catalyzed PUFAs Oxygenation

Since LOX activity is conventionally measured with PUFA substrates,^{14,18)} we initially tested the inhibitory effects of CBDD (Fig. 1B), as well as several structurally related compounds including NDGA, a well-characterized pan-LOX inhibitor, on 15-LOX-catalyzed oxygenation of arachidonic acid (AA), by measuring the formation of hydroperoxides. The compounds tested in our investigation have previously been established as inhibitors of 15-LOX-catalyzed linoleic acid (LA) oxygenation.^{15,16)} With the exception of CBDD, the other compounds contain reducing moieties, such as catechol, resorcinol, and phenol in their respective structures.^{15,16)} LOX enzymes require the ferric state (Fe^{3+}) to initiate their enzymatic reaction.¹⁷⁾ As expected, $5\text{ }\mu\text{M}$ NDGA significantly inhibited 15-LOX activity. Although a resorcinol ring-containing CBD potently inhibited 15-LOX-catalyzed LA oxygenation ($IC_{50}=2.56\text{ }\mu\text{M}$), possibly *via* direct inhibition and reduction of Fe^{3+} into Fe^{2+} (ferrous state) in the active center of 15-LOX,¹⁷⁾ the enzymatic reaction of AA was only poorly inhibited by this agent at $5\text{ }\mu\text{M}$. At $50\text{ }\mu\text{M}$, CBD, CBDD, and Δ^9 -THC all significantly inhibited the activity of the enzyme (Fig. 2A). Although at $5\text{ }\mu\text{M}$ both CBDD and Δ^9 -THC exhibited strong inhibitory effects on 15-LOX, CBDD, whose resorcinolic OH groups are dimethylated, exhibited more potent efficacy than CBD, a prototype of the dimethylated form

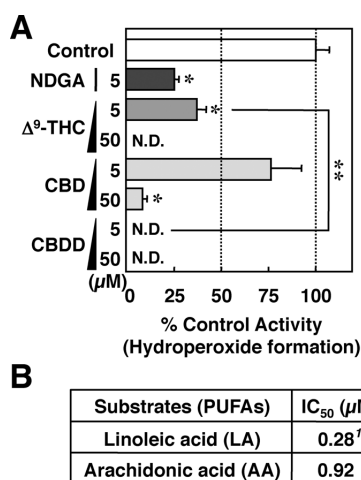


Fig. 2. Effects of NDGA, Δ^9 -THC, CBD, and CBDD on 15-LOX-Mediated Formation of Arachidonic Acid Hydroperoxides

(A) Enzymatic reactions were initiated with arachidonic acid (AA), and then chromogen was added to stop the reactions and to develop the colorimetric reaction product. The absorbance was monitored at 490 nm. The assay conditions are described in Materials and Methods. Each bar represents the mean \pm S.D. (triplicate determinations) of the relative activity compared to control. * Significantly different ($p < 0.05$) from control. ** Significantly different ($p < 0.05$) from the 5 μ M CBDD-treated activity. N.D., Not detectable (because of complete inhibition). (B) Comparison of IC₅₀ values for CBDD-mediated inhibition of 15-LOX when using an AA (this report) or linoleic acid (LA) as a substrate.¹⁵⁾ The former IC₅₀ value was determined by varying AA concentrations from 0.1 to 5 μ M.

(Fig. 2A). The IC₅₀ values for the inhibition of 15-LOX by CBDD and CBD were determined as 0.92 μ M (Fig. 2B) and 12.38 μ M, respectively, suggesting that CBDD inhibits the formation of AA hydroperoxides catalyzed by 15-LOX in addition to LA. Further, 5-LOX-mediated oxygenation of AA was not inhibited by CBDD, even at 50 μ M concentrations (data not shown). We also used an alternative methodology to assess the inhibitory effect of CBDD on PUFAs, measuring the effect of CBDD on the formation of conjugated dienes on LA or AA during the oxygenation by 15-LOX, as compared with CBD. In these assays, 15-LOX preferably catalyzed the formation of conjugated dienes of LA over that of AA (Fig. 3A). The differences in the measured CBDD-mediated IC₅₀ values between LA (0.28 μ M) and AA (0.92 μ M) (Fig. 2B) could perhaps be due to differences in the substrate specificity of 15-LOX that involve LA *versus* AA interactions or alignment within the active site(s) of the enzyme. In these respects, it has also been suggested that 15-LOX enzymes possess allosteric binding site(s) in the structure,^{14,21)} hence allosterism may also account for the differences noted.

Interestingly, as demonstrated in Fig. 3B, the inhibition potential by 1 μ M CBDD and CBD on 15-LOX-catalyzed oxygenation of LA (panel a) and AA (panel b), was closely comparable. In general, LOX-catalyzed oxygenation of PUFAs consists of four consecutive reactions: i) hydrogen abstraction, ii) radical rearrangement (conjugated dienes), iii) oxygen insertion (lipid peroxy radicals), and iv) peroxy radical reduction (hydroperoxides).¹⁴⁾ The peroxy radical formed in step iii) is known to be highly reactive and is capable of propagating the chain reaction.¹⁴⁾ Given the data that CBDD more potently inhibits 15-LOX-catalyzed hydroperoxide formation of PUFAs as compared to CBD (CBDD: IC₅₀=0.28 μ M for LA; IC₅₀=0.92 μ M for AA; CBD: IC₅₀=2.56 μ M for LA; IC₅₀=12.38 μ M for AA), it is therefore suggested that

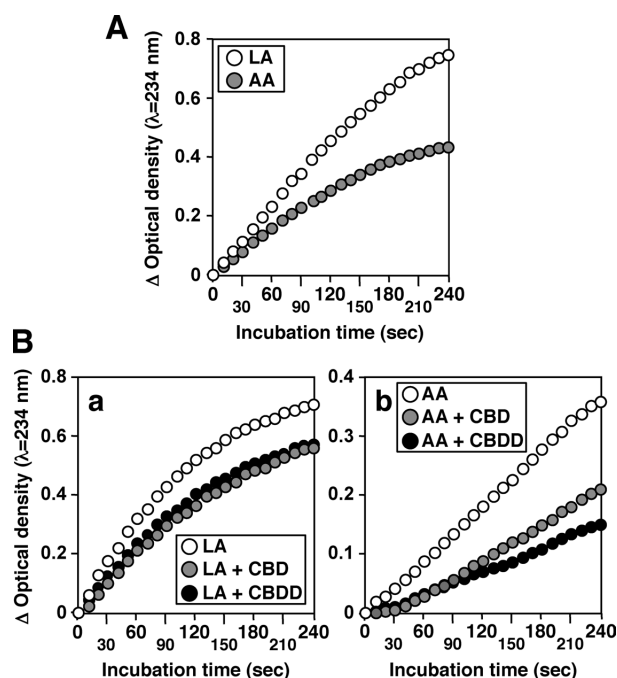


Fig. 3. Effects of CBDD and CBD on 15-LOX-Mediated Formation of Conjugated Dienes on PUFAs

(A) PUFAs (LA or AA) were incubated with 15-LOX for up to 240 s as described in Materials and Methods. Absorbance at 234 nm was periodically recorded. Results are expressed as an increase in absorbance from the zero time reaction mixture. (B) PUFAs (LA, panel B-a or AA, panel B-b) were incubated with 15-LOX in the presence of 1 μ M CBDD or CBD for up to 240 s as described in Materials and Methods. Absorbance at 234 nm was periodically recorded. Results are expressed as an increase in absorbance from the zero time reaction mixture.

CBDD more specifically and/or more potently inhibits the reaction step iii) and/or iv) compared with CBD. Further, it appears reasonable to suggest that the CBD-mediated inhibition of 15-LOX might be modified by methylation of the resorcinolic OH groups, leading to gains in CBDD inhibitory selectivity to reaction steps iii) and/or iv). Further studies are required to clarify these exact mechanisms.

Taken together, the results obtained in this investigation firmly demonstrate that CBDD is a potent inhibitor of 15-LOX-catalyzed PUFAs oxygenation, likely *via* its inhibition of PUFA hydroperoxide formation.

CBDD Interferes with 15-LOX-Mediated CL Oxidation Since it is reported that 15-LOX effectively targets cholesteryl linoleate (CL) as a substrate,^{9,11,13)} and that the CL is the most abundant substrate for oxidation within the LDL,^{19,13)} we focused on our subsequent studies to assess the effects of 10 μ M CBDD and CBD on 15-LOX-mediated oxygenation of CL. At “1 μ M” concentrations of the cannabinoids, no remarkable inhibitory effects of CBDD and CBD on the reaction were observed (data not shown) (see also the results described in Fig. 5A, lanes 10–12). It was evident that CBDD was a much more potent inhibitor of the reaction than CBD (Fig. 4). However, the inhibition profile between CBDD and CBD differed in that CBD effectively squelched the early oxidation phase of the reaction, for approximately 90 s, whereas CBDD addition resulted in a sustained and marked inhibition of the reaction. These data demonstrate that CBDD effectively abrogates 15-LOX-catalyzed CL oxygenation.

We noted that CBDD was a more efficacious inhibitor than

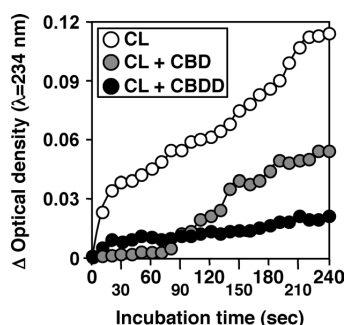


Fig. 4. Effects of CBDD and CBD on 15-LOX-Mediated Formation of Conjugated Dienes on Cholesteryl Linoleate (CL)

CL was incubated with 15-LOX in the presence of 10 μ M CBDD or CBD for up to 240 s as described in Materials and Methods. Absorbance at 234 nm was periodically recorded. Results are expressed as an increase in absorbance from the zero time reaction mixture.

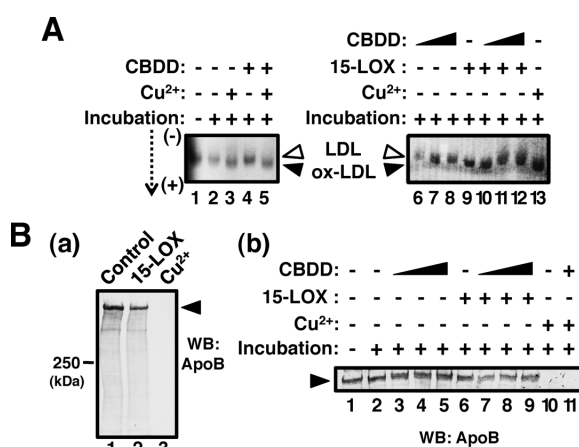


Fig. 5. Effect of CBDD on Oxidation of LDL by Cu^{2+} (Non-enzymatic) or 15-LOX (Enzymatic)

(A) Agarose gel electrophoresis of LDL incubated with Cu^{2+} or 15-LOX in the presence (+) or absence (−) of CBDD at 37°C for 5 h. Lane 1, 0 h (no incubation); lane 2, 5 h (control incubation for 5 h); lane 3, Cu^{2+} ; lane 4, 10 μM CBDD; lane 5, 10 μM CBDD/ Cu^{2+} ; lanes 6–8, 1, 10, or 100 μM CBDD, respectively; lane 9, 15-LOX; lanes 10–12, 15-LOX/1, 10, or 100 μM CBDD, respectively; lane 13, Cu^{2+} . Empty arrowhead indicates LDL and filled arrowhead indicates ox-LDL in the figure. (B) Western blot (WB) analysis of LDL incubated with Cu^{2+} or 15-LOX at 37°C for 5 h, using an antibody specific for human apoB protein. (a) Lane 1, 5 h (control incubation for 5 h); lane 2, 15-LOX; lane 3, Cu^{2+} . Filled arrowhead indicates apoB (Molecular Weight (MW) *ca.* 500 kDa), respectively. (b) Lane 1, 0 h (no incubation); lane 2, 5 h (control incubation for 5 h); lanes 3–5, 1, 10, or 100 μM CBDD, respectively; lane 6, 15-LOX; lanes 7–9, 15-LOX/1, 10, or 100 μM CBDD, respectively; lane 10, Cu^{2+} ; lane 11, 10 μM CBDD/ Cu^{2+} .

CBD when CL (–COO–cholesterol) was used as a substrate, but not LA (–COOH). To better understand the potential mechanistic basis for this difference, we first recognized that esters are much more lipophilic than their respective carboxylic acids. In this respect, we performed an *in silico* docking simulation study examining CBDD:15-LOX *versus* CBD:15-LOX interactions. Interestingly, the analysis suggested that the much greater lipophilicity of CBDD was likely an important determinant inhibitory efficacy for 15-LOX, resulting from stronger hydrophobic interactions with amino acid residues within the 15-LOX binding pocket (Takeda and Fukunishi *et al.*, unpublished observations).

CBDD Interferes with 15-LOX-Mediated ox-LDL Formation It is known that LDL can be oxidized enzymatically or non-enzymatically.^{1,8,12)} The later reaction is mediated by Cu²⁺-catalyzed oxidation.⁸⁾ A major goal of this

study was to assess whether CBDD can inhibit 15-LOX-dependent production of ox-LDL formation, since PUFAs, especially LA, are composed as cholesteryl esters in the LDL (see Fig. 1A). It is established that oxidative modification of LDL results in a greater electrophoretic mobility on agarose gels, since the net negative charge on LDL is increased when oxidized into ox-LDL.^{8,22} Based on this phenomenon, we first studied the effect of CBDD on Cu²⁺-mediated ox-LDL formation. Ox-LDL formation was stimulated by 2 μM Cu²⁺, while 10 μM CBDD failed to exert any detectable effect on ox-LDL formation (Fig. 5A, lanes 3 vs. 5). The absence of CBDD's effect on this pathway was similarly observed even at 100 μM concentrations of CBDD (data not shown), as in the 10 μM CBDD-treated samples shown in Fig. 5A (lane 4). We next investigated the effect of CBDD on 15-LOX-mediated ox-LDL formation. 15-LOX also catalyzed formation of ox-LDL (Fig. 5A, lane 9), at a level closely comparable to that of Cu²⁺-mediated ox-LDL formation (Fig. 5A, lanes 9 vs. 13). When compared to the results seen for Cu²⁺/CBDD (Fig. 5A, lanes 3 vs. 5), CBDD specifically inhibited 15-LOX-mediated ox-LDL formations in a concentration-dependent manner (Fig. 5A, lanes 10–12; 1, 10, and 100 μM , respectively), although 1 μM CBDD could not abrogate the formation of ox-LDL (Fig. 5A, lane 10). Taken together with the results demonstrated in Fig. 4, it is suggested that concentration requirements for the CBDD-mediated interference on 15-LOX-catalyzed oxygenation of CL or LDL may be dependent upon the substrates of 15-LOX. It should be noted that CBDD additions themselves did not cause any changes in LDL migration (Fig. 5A, lanes 4, 6–8).

Further, using after the treatments with 15-LOX or Cu^{2+} , the intactness of apolipoprotein B (apoB), the most abundant protein in LDL (see Fig. 1A), was studied by Western blot analysis. ApoB protein can undergo striking degradation,²³⁾ especially by free radical generated in Cu^{2+} -mediated oxidation reactions.⁸⁾ As demonstrated in Fig. 5B(a), the apoB protein band completely disappeared in the presence of Cu^{2+} , but not by 15-LOX, suggesting that apoB in LDL is quite sensitive to Cu^{2+} -mediated oxidation. The 15-LOX-mediated oxidation of LDL appears relatively mild, without marked effect on apoB protein levels. In support of this result, Sparrow *et al.* reported that 15-LOX-mediated degradation of apoB also requires the presence of phospholipase A_2 .²³⁾ Given that the CBDD-mediated inhibition of ox-LDL formation is attributed to inhibition of the 15-LOX enzyme, the degradation of apoB by Cu^{2+} should not be effected by CBDD. In support of this idea, no protection was provided by CBDD additions (Fig. 5B(b), lanes 10 vs. 11). Further, no observable effects of 1–100 μM CBDD alone or in combination with the 15-LOX enzyme were detected (Fig. 5B(b), lanes 3–5 and 7–9, respectively). As is apparent in Fig. 5A, intensities of the stained CBB band varied from lane 1 to lane 13. We suggest that the variable staining intensities likely reflect protein components in the LDL/ox-LDL, as the LDL and ox-LDL were also stained, with similar migration patterns, by Oil Red O dye which stains lipids in the lipoproteins (data not shown). Taken together, these data strongly suggest that CBDD selectively interferes with enzymatic formation of ox-LDL as catalyzed by 15-LOX, but not with the non-enzymatic formation of ox-LDL from Cu^{2+} -mediated oxidation.

In general, cannabinoids evoke their pharmacological ac-

tions *via* engagement with cannabinoid receptors (CB1 or CB2).^{24,25} Especially, agonists for the CB1 receptor are known to possess psychoactive properties, which can be considered adverse effects.^{24,25} If CBDD behaves as an agonist for CB1 receptor, these actions might limit the clinical utility of the compound. However, CBD is established as a non-psychoactive cannabinoid and exhibits little binding affinity for CB receptors,^{24–26} and similarly, it has been shown that CBDD has no binding affinity for the receptor.²⁶ Further, although CBD can be converted into a cannabielsoin when incubated with guinea pig liver microsomes that contain drug-metabolizing enzymes including P450s,^{19,20,27–30} CBDD is resistant to the liver microsome-mediated metabolism.²⁷ Therefore, it appears that CBDD offers a potentially positive safety profile, and that CBDD-mediated inhibition of 15-LOX may be retained in *in vivo* situations.

In sum, these findings suggest that CBDD may be a useful adjuvant in the treatment of atherosclerosis as well as an experimental tool for analyzing the mechanistic details of PUFAs oxygenation by 15-LOX. Further studies will be required to assess the potential *in vivo* effects of CBDD.

Acknowledgements We thank Ms. Satomi Abe for technical assistance. This study was supported by Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan [Research Nos. 20790149 and 22790176 (S.T.)]. This study was also supported by the donation from NEUES Corporation, Japan (H.A.). C.J.O. was supported by a USPHS award, ES016358. A part of this work was performed as part of the NEDO project, Development of Basic Technology for Protein Structure Analysis Aimed at Acceleration of Drug Discovery Research, directed by Prof. Haruki Nakamura at the Institute for Protein Research, Osaka University.

REFERENCES

- 1) Funk C. D., *Biochim. Biophys. Acta*, **1304**, 65–84 (1996).
- 2) Kühn H., Belkner J., Zaiss S., Fährenklemper T., Wohlfeil S., *J. Exp. Med.*, **179**, 1903–1911 (1994).
- 3) Paoletti R., Gotto A. M. Jr., Hajjar D. P., *Circulation*, **109**, III-20–III-26 (2004).
- 4) Ishigaki Y., Katagiri H., Gao J., Yamada T., Imai J., Uno K., Hasegawa Y., Kaneko K., Ogihara T., Ishihara H., Sato Y., Takikawa K., Nishimichi N., Matsuda H., Sawamura T., Oka Y., *Circulation*, **118**, 75–83 (2008).
- 5) Rydberg E. K., Krettek A., Ullström C., Ekström K., Svensson P. A., Carlsson L. M., Jönsson-Rylander A. C., Hansson G. I., McPheat W., Wiklund O., Ohlsson B. G., Hultén L. M., *Arterioscler. Thromb. Vasc. Biol.*, **24**, 2040–2045 (2004).
- 6) Cyrus T., Witztum J. L., Rader D. J., Tangirala R., Fazio S., Linton M. F., Funk C. D., *J. Clin. Invest.*, **103**, 1597–1604 (1999).
- 7) Harats D., Shaish A., George J., Mulkins M., Kurihara H., Levkovitz H., Sigal E., *Arterioscler. Thromb. Vasc. Biol.*, **20**, 2100–2105 (2000).
- 8) Tanaka K., Iguchi H., Taketani S., Nakata R., Tokumaru S., Sugimoto T., Kojo S., *J. Biochem.*, **125**, 173–176 (1999).
- 9) Upston J. M., Neuzil J., Witting P. K., Alleva R., Stocker R., *J. Biol. Chem.*, **272**, 30067–30074 (1997).
- 10) Arai H., Suzuki T., Takama K., Terao J., *Biochem. Biophys. Res. Commun.*, **228**, 675–682 (1996).
- 11) Folcik V. A., Nivar-Aristy R. A., Krajewski L. P., Cathcart M. K., *J. Clin. Invest.*, **96**, 504–510 (1995).
- 12) Takahashi Y., Yoshimoto T., *Res. Adv. Cancer*, **2**, 221–229 (2002).
- 13) Scanu A. M., “The Biochemistry of Atherosclerosis,” ed. by Scanu A. M., Marcel Dekker, Inc., New York, 1979, pp. 3–8.
- 14) Ivanov I., Heydeck D., Hofheinz K., Roffeis J., O'Donnell V. B., Kuhn H., Walther M., *Arch. Biochem. Biophys.*, **503**, 161–174 (2010).
- 15) Takeda S., Usami N., Yamamoto I., Watanabe K., *Drug Metab. Dispos.*, **37**, 1733–1737 (2009).
- 16) Takeda S., Jiang R., Aramaki H., Imoto M., Toda A., Eyanagi R., Amamoto T., Yamamoto I., Watanabe K., *J. Pharm. Sci.*, **100**, 1206–1211 (2011).
- 17) Kemal C., Louis-Flamberg P., Krupinski-Olsen R., Shorter A. L., *Biochemistry*, **26**, 7064–7072 (1987).
- 18) Cathcart M. K., McNally A. K., Chisolm G. M., *J. Lipid Res.*, **32**, 63–70 (1991).
- 19) Takeda S., Ishii Y., Iwanaga M., Nurrochmad A., Ito Y., Mackenzie P. I., Nagata K., Yamazoe Y., Oguri K., Yamada H., *Mol. Pharmacol.*, **75**, 956–964 (2009).
- 20) Takeda S., Kitajima Y., Ishii Y., Nishimura Y., Mackenzie P. I., Oguri K., Yamada H., *Drug Metab. Dispos.*, **34**, 1277–1282 (2006).
- 21) Mogul R., Johansen E., Holman T. R., *Biochemistry*, **39**, 4801–4807 (2000).
- 22) Puhl H., Waeg G., Esterbauer H., *Methods Enzymol.*, **233**, 425–441 (1994).
- 23) Sparrow C. P., Parthasarathy S., Steinberg D., *J. Lipid Res.*, **29**, 745–753 (1988).
- 24) Howlett A. C., Barth F., Bonner T. I., Cabral G., Casellas P., Devane W. A., Felder C. C., Herkenham M., Mackie K., Martin B. R., Mechoulam R., Pertwee R. G., *Pharmacol. Rev.*, **54**, 161–202 (2002).
- 25) Izzo A. A., Borrelli F., Capasso R., Di Marzo V., Mechoulam R., *Trends Pharmacol. Sci.*, **30**, 515–527 (2009) [Erratum in *Trends Pharmacol. Sci.*, **30**, 609 (2009)].
- 26) Thomas A., Ross R. A., Saha B., Mahadevan A., Razdan R. K., Pertwee R. G., *Eur. J. Pharmacol.*, **487**, 213–221 (2004).
- 27) Gohda H., Narimatsu S., Yamamoto I., Yoshimura H., *Chem. Pharm. Bull.*, **38**, 1697–1701 (1990).
- 28) Takeda S., Ishii Y., Mackenzie P. I., Nagata K., Yamazoe Y., Oguri K., Yamada H., *Biol. Pharm. Bull.*, **28**, 2026–2027 (2005).
- 29) Takeda S., Ishii Y., Iwanaga M., Mackenzie P. I., Nagata K., Yamazoe Y., Oguri K., Yamada H., *Mol. Pharmacol.*, **67**, 665–672 (2005).
- 30) Ishii Y., Takeda S., Yamada H., *Drug Metab. Rev.*, **42**, 145–158 (2010).