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Cannabidiolic Acid as a Selective Cyclooxygenase-2 Inhibitory Component in Cannabis

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Running title page

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d) ABBREVIATIONS: CBDA, cannabidiolic acid; COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine; Δ^9-THC, Δ^9-tetrahydrocannabinol, CBD, cannabidiol.

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ABSTRACT:

In the present study it was revealed that cannabidiolic acid (CBDA) selectively inhibited cyclooxygenase-2 (COX-2) activity with an IC_{50} value (50% inhibition concentration) around 2 μM, having 9-fold higher selectivity than COX-1 inhibition. In contrast, Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) was a much less potent inhibitor of COX-2 (IC_{50} > 100 μM). Nonsteroidal anti-inflammatory drugs containing a carboxyl group in their chemical structures such as salicylic acid are known to inhibit nonselectively both COX-1 and COX-2. CBDA and Δ^9-THCA have a salicylic acid moiety in their structures. Thus, the structural requirements for the CBDA-mediated COX-2 inhibition were next studied. There is a structural difference between CBDA and Δ^9-THCA; phenolic hydroxyl groups of CBDA are freed from the ring formation with the terpene moiety, although Δ^9-THCA has dibenzopyran ring structure. It was assumed that the whole structure of CBDA is important for COX-2 selective inhibition, since β-resorcylic acid itself did not inhibit COX-2 activity. Methylation of the carboxylic acid moiety of CBDA led to disappearance of COX-2 selectivity. Thus, it was suggested that the carboxylic acid moiety in CBDA is a key determinant for the inhibition. Further, the crude extract of cannabis containing mainly CBDA was shown to have a selective inhibitory effect on COX-2. Taken together, these lines of evidence in this study suggest that naturally occurring CBDA in cannabis is a selective inhibitor for COX-2.

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Introduction

Cannabis is one of the oldest known medicinal plants and produces pharmacologically important substances. Among them, most important are the cannabinoids that are unique components in the cannabis plant. Δ^9-Tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are known to be major cannabinoids in the plant. Δ^9-THC is known to have pharmacological effects such as psychoactivity and hallucination (Dewey, 1986; Howlett et al., 2002) Cannabinoids (i.e., Δ^9-THC and CBD) are also being used as a rheumatoid arthritis agent in clinical settings (Klein and Newton, 2007) because of their anti-inflammatory effects (Formukong et al., 1998). In fresh plant materials, most of Δ^9-THC and CBD exist as their respective acid forms, Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabidiolic acid (CBDA) (Yamauchi et al., 1967; Turner et al., 1980; Taura et al., 2007). The specific use of acidic cannabinoids including Δ^9-THCA and CBDA, as the active pharmaceutical ingredients, is not disclosed to date since these acid forms of cannabinoids are recognized as pharmacologically inactive forms (Yamauchi et al., 1967; Razdan, 1986; Burstein, 1999). By focusing on the structures between Δ^9-THCA and CBDA, it was revealed that both acidic cannabinoids have a salicylic acid moiety in their structures (Fig. 1). Salicylic acid is known to be an inhibitor of cyclooxygenases

(COXs, also referred as prostaglandin H synthases). Most of the conventional

COX-1 and/or nonselective inhibitors contain a carboxylic acid group in their structures, and the COX-2 selective inhibitors reported lack the acid group but contain a sulfonyl-like group.

COX, which exists in at least two isoforms, catalyzes the first key steps in the synthesis of all prostaglandins (PGs) by converting arachidonic acid (AA) into PGH_2. Thus, COX is a bifunctional enzyme exhibiting both cyclooxygenase (from AA to PGG_2) and peroxidase (from PGG_2 to PGH_2) activities (DeWitt, 1999; Hinz and Brune, 2002). COX-1 is constitutively expressed as a housekeeping enzyme in nearly all tissues, and mediates physiological responses (e.g., cytoprotection of the stomach, platelet aggregation). On the other hand, COX-2 is expressed by cells that are involved in inflammation and has emerged as the isoform primarily responsible for the synthesis of prostanoids involved in acute and chronic inflammatory states of pathological processes (DeWitt, 1999; Hinz and Brune, 2002). Classical nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (Aspirin) and diflunisal, which are grouped into the salicylates derivatives of NSAIDs, were shown to inhibit both COX-1 and COX-2 activities (DeWitt, 1999; Warner et al., 1999). None of the COX-2 selective inhibitors belonging to salicylates, which show selectivity for COX-2 inhibition with low concentrations, are reported to date (DeWitt, 1999; Warner et al., 1999). Inhibition of COX-2-dependent PG synthesis accounts for the anti-inflammatory and analgesic effects of NSAIDs, while suppression of COX-1 can lead to many

unwanted side effects (e.g., gastrointestinal ulceration and bleeding, platelet dysfunctions). Thus, it has been thought that specific inhibitors for the COX-2-mediated reaction might have ideal therapeutic actions similar to those of classical NSAIDs without causing adverse effects. Burstein et al. have reported that some of natural cannabinoids inhibited PGE synthesis in bull seminal vesicles (Burstein et al., 1973). However, there is no report whether any cannabinoid(s) selectively inhibit the COX-2 isoform.

The present report describes that CBDA is a selective COX-2 inhibitor in cannabis. The mechanism of selective COX-2 inhibition by CBDA is discussed.

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Materials and Methods

Cannabinoids and Chemicals. Cannabis leaves were harvested from *Cannabis sativa L.* of Δ^9-THCA (Mexican) and CBDA strains grown in the botanical garden of Hokuriku University. Δ^9-THC, Δ^9-THCA, CBD, and CBDA were isolated and purified from the cannabis leaves according to the methods described elsewhere (Aaramaki et al., 1968). Purities of these cannabinoids were checked to be at least 95%–98% by gas chromatography (GC) (Watanabe et al., 2005). The crude extract from CBDA strain was prepared by the methods described previously (Watanabe et al., 2005) except that the crude extract was not treated with heating to decompose the acid forms (i.e., decarboxylation). In fresh plant material, most of CBD has been reported to exist as its acid form (Turner et al., 1980). The relative contents of CBDA (77%) and CBD (23%) were determined by thin-layer chromatography analysis using Fast Blue BB salt as a coloring reagent (Watanabe et al., 2005). To obtain more information GC analysis was employed. In GC analysis, since the applied CBDA in cannabis is subject to heating conditions causing its decomposition into CBD (~100%), the apparent content of CBDA in the strain was determined as CBD. The extract from CBDA strain contained 0.22 mg/mL of CBD. The content of Δ^9-THC in the extract of CBDA strain was not determined because Δ^9-THC concentration was less than the detection limit (≤0.01 mg/mL). CBDA methyl ester was prepared by the

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methylation of CBDA with diazomethane (Watanabe et al., 1988). 2,4-Dihydroxybenzoic acid (β-resorcylic acid), indomethacin, and resorcinol were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Diclofenac was purchased from Sigma (St Louis, MO). All other reagents were of analytical grade.

Enzyme Sources. Assay of recombinant COX-1 and COX-2 activity was performed by using a commercially available colorimetric COX (ovine) inhibitor screening assay kit (Cayman Chemical Company, Ann Arbor, MI; lot nos. 184104). All inhibitors added to the reaction system were dissolved in ethanol and prepared just before use. In this assay, the COX activity was measured by utilizing N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) as a co-substrate with AA (reduction of PGG_2 to PGH_2). TMPD oxidation was monitored spectrophotometrically with a 96-well plate reader at 590 nm. No colorimetric change was observed in control incubations that were performed by omitting enzymes or with heat denatured enzymes and inhibitors in combination with TMPD.

Data Analysis. The concentration of the inhibitor that is required to produce 50% inhibition of the enzymatic activity (IC_{50}) was determined from the curves plotting enzymatic activity versus inhibitor concentrations using Origin7.5J software (OriginLab Corp., Northampton, MA). The details of the calculations were described in our previous paper (Takeda et al., 2006; Yamaori et al., 2007).

Differences were considered to be significant when the *p* value was calculated to be less than 0.05. All statistical analyses were performed by Scheffe's F test, which is a type of post-hoc test for analyzing results of ANOVA testing. These calculations were done using Statview5.0J software (SAS Institute Inc., Cary, NC).

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Results

Effects of Cannabinoids on COX Activity. The inhibitory effects of Δ^9-THC and CBD, and their respective acid forms Δ^9-THCA and CBDA on COX-mediated TMPD oxidation activity were examined using purified COX as enzyme sources. Although COX-1 activity was not significantly inhibited by the addition of 100 μM of Δ^9-THC, Δ^9-THCA, or CBD except for CBDA, COX-2 activity was strongly inhibited by CBDA treatment compared to Δ^9-THCA (around 10%)(Fig. 2). NSAIDs used in this study (indomethacin and diclofenac) nonselectively inhibited COX-1/-2 (see also Table 1). Further, it should be noted that although both Δ^9-THCA and CBDA have the same structural moiety; namely, salicylic acid (Fig. 1), the inhibition potency between these two acids was quite different. Based on the results obtained in Fig. 2, the following experiments focused on the inhibition potential of CBDA for COX-2 activity. To obtain a selectivity index (COX-1/COX-2 ratio of IC_{50} values), we next determined IC_{50} values for the inhibition of the two COX isoforms by CBDA. Although CBDA inhibited both COX-1 and COX-2, with apparent IC_{50} values of 20 ± 1.5 and 2.2 ± 0.3, respectively (Fig. 3A and 3B), it was revealed that CBDA was a selective inhibitor for COX-2 based on its selectivity index of 9.1 (i.e., >1)(Fig. 3 and Table 1).

Structural Requirement for Inhibitory Effect of CBDA on COXs Activity. To determine key structural determinants of CBDA-mediated COX-2 selective

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inhibition we performed structure-activity relationship analysis. Interestingly, β-resorcylic acid only significantly inhibited COX-1 activity, although resorcinol equipotently inhibited COX enzymes (Fig. 4A). These structural components of CBDA were added to the reaction system at 2 μM as well as CBDA based on the IC_{50} value for COX-2 inhibition of CBDA (Fig. 3B). Thus, we hypothesized that β-resorcylic acid moiety of CBDA is one of the key structures of CBDA-mediated inhibition of COXs (COX-2), although β-resorcylic acid itself is insufficient for selective inhibition of COX-2. We discussed this point in the discussion section. We next studied the effect of the methyl ester form of CBDA on COX-2 activity (Fig. 4B). The result indicated that the free carboxylic acid portion of CBDA is important to express its full inhibitory activity. Collectively, it was revealed that CBDA is able to inhibit COX-2 activity, which relies on the β-resorcylic acid moiety whose 6'-hydroxyl residue has to be freed from ring formation with the terpene moiety (see the structure of Δ^9-THCA in Fig. 1).

Effect of the Crude Extract from CBDA Strain Cannabis on COX Activity. This study was performed to investigate the possibility that CBDA is a COX-2 specific inhibitory component in cannabis; the inhibition by CBDA would be also seen even in the presence of other constitutive components in cannabis. The extract from CBDA strain, which contains CBDA as a major cannabinoid, inhibited both COX-1 and COX-2 activities at a concentration of 37.5 μg/mL (25 μM in terms of CBDA concentration)(Fig. 5) although this inhibitory effect was not

observed at a concentration of 7.5 μg/mL (5 μM in terms of CBDA concentration)(Fig. 5). The inhibitory magnitude of COX-2 by the extract from CBDA strain cannabis was clearly higher than that of COX-1. Therefore, CBDA itself is suggested to be a selective COX-2 inhibitory component in cannabis. However, it was also revealed that the inhibitory potency of CBDA in the extract might be much weaker than that of pure CBDA (Figs. 3 and 5). We discussed this inconsistency in the Discussion Section.

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Discussion

Although it was considered that cannabinoid acids in cannabis plant were inactive cannabinoids, in the present study it was revealed that CBDA is a selective COX-2 inhibitor in vitro (selectivity index = 9.1; Table 1). Burstein et al. reported that several cannabinoids were able to suppress the biosynthesis of PGE in bull seminal vesicles, with large IC_{50} values ranging from 70 to 300 μM (Burstein et al., 1973). Since COX-2 is basically inducible by stimulations (DeWitt, 1999; Hinz and Brune, 2002), it is reasonable to understand that they only focused on the relationship between COX-1 and cannabinoids investigated. In agreement with their report, Δ^9-THC was also a very weak inhibitor for COX-1 in our experiments (IC_{50} value; > 100 μM)(Fig. 2). After the discovery of COX-2 (Kujubu et al., 1991; O'Banion et al., 1991; Xie et al., 1991) it became a therapeutic target to avoid side effects by nonselective COX inhibitors. Thus, we set out to discover constituent(s) that have COX-2 selectivity in cannabis, and then CBDA was found to be an inhibitor for COX-2 (Figs. 2 and 3). Although Δ^9-THC and CBD have been reported to have potential use as an analgesic for patients suffering from rheumatoid arthritis (Klein and Newton, 2007), it has been suggested that the anti-inflammatory effect of Δ^9-THC and CBD is not mediated by COX enzymes inhibition (Russo and Guy, 2006). Thus, it is assumed that the inhibition mechanism between Δ^9-THC/CBD and CBDA in anti-inflammation is different. However, including this possibility, we are left with further questions that

we were not able to address in these studies such as, does CBDA have potential to inhibit the COX-2-mediated PGs production, which is able to lead to an anti-inflammatory action *in vivo*? A study about this possibility is under investigation.

It is well known that NSAIDs (salicylates) with an acidic carboxylic acid moiety, such as diflunisal and salicylic acid, inhibit COX activity via forming a salt bridge with Arg120 in COX enzymes (Picot et al., 1994; Mancini et al., 1995; Kurumbail et al., 1996; Luong et al., 1996). Thus, cannabinoids containing a carboxylic acid residue in their structures (i.e., both Δ^9-THCA and CBDA) were expected to be effective COX inhibitors (Fig. 1). However, this does not seem to be the case with Δ^9-THCA (Fig. 2). There is only one structural difference between these; namely, the 6'-hydroxyl group of CBDA is freed from the ring formation with the terpene moiety. Based on this information, to obtain further experimental evidence we studied the effects of the structural moieties of CBDA, resorcinol and β-resorcylic acid, on COX activity. As expected, both COX-1 and COX-2 activities were inhibited by a reducing agent (i.e., antioxidant) resorcinol, because it has been reported that the COX activity is sensitive to a large number of reducing agents which act as reducing co-substrate for peroxidase reaction of COX enzymes (Markey et al., 1987). On the other hand, COX-1 but not COX-2 was significantly inhibited by β-resorcylic acid, a non-reducing agent (Seeram et al., 2001)(Fig. 4A). It should also be noted here that COX-2 activity was inhibited by CBDA itself,

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but COX-1 was not inhibited (Fig. 4). It appears that the inhibitory effects of pure CBDA and the crude extract are different (Figs. 3 and 5). The reason for this discrepancy is not clear at present, although there is a possibility that CBDA crude extract contains other interfering component(s) for attenuating CBDA-mediated COX-2 inhibition. The X-ray crystal structures of the COX-1 and COX-2 enzymes have presented insight into how COX-2 specificity is achieved. Within the hydrophobic channel of the COX proteins, a single amino acid difference in position 523 (isoleucine in COX-1, valine in COX-2) has been shown to be critical for the COX-2 selectivity (Hood et al., 2003). Thus, the total NSAIDs-binding site is around 17% larger in the COX-2 isoform (Luong et al., 1996), which allows COX-2 to bind bulky inhibitors more readily than COX-1 (Kurumbail et al., 1996). Although the results obtained here (Fig. 4A) are complex, at least two possibilities might be that i) β-resorcylic acid itself can enter the catalytic site of both COX enzymes because of its smaller molecular size compared to that of CBDA and ii) the whole molecule of CBDA is fitted by ideal configuration(s) with COX-2, which leads to COX-2 selective inhibition via its carboxylic acid moiety (see also Fig. 4B). Since there are no structural similarities between CBDA and celecoxib, a highly selective COX-2 inhibitor (selectivity index = 60.48; Table 1; see also Fig. 1), we propose the possibility that CBDA will be a useful ‘prototype’ for producing COX-2 selective inhibitors different from celecoxib.

Taking all these findings into consideration, we have shown that CBDA in cannabis is a potent and selective inhibitor for COX-2 *in vitro*. Further studies are necessary to obtain information about molecular mechanism of the inhibition.

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Footnotes

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Legends for figures

FIG. 1. Structures of cannabinoids tested and celecoxib.

FIG. 2. Effects of cannabinoids on COX activity. CBDA was a potent inhibitor for COX-2. Reactions were initiated with arachidonic acid, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control. *significantly different ($p < 0.05$) from control. #significantly different ($p < 0.05$) from Δ^9-THC, Δ^9-THCA, and CBD-treated groups. N.S., not significant.

FIG. 3. Dose-dependent inhibition by CBDA on COX activity. (A) and (B) TMPD oxidation by two isoforms of COX enzyme (A, COX-1; B, COX-2) was examined in the presence of indicated concentrations of CBDA. Panel (B) is composed of two parts; left is high concentration range (2.5 – 100 μM), right is low concentration range (0.1 – 100 μM). Reactions were initiated with arachidonic acid, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods.* Each plot represents the mean ± S.D. (triplicate determinations) of the relative activity to the control.

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FIG. 4. Structural requirement of CBDA-mediated COX-2 selective inhibition. (A) Effects of structural moieties of CBDA (resorcinol and β-resorcylic acid) on the COX activities. These were added to the reaction mixture at 2 μM (determined on the basis of IC_{50} value for the COX-2 inhibition of CBDA; see Fig. 3B) and their structures are shown. (B) Effect of CBDA methyl ester (CBDA-Me) on the COX-mediated TMPD oxidation. Reactions were initiated with arachidonic acid, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods.* Each bar represents the mean ± S.D. (triplicate determinations) of the relative activity to the control. *significantly different compared with the control group ($p < 0.05$).

FIG. 5. Effect of the crude extract from CBDA strain on COX activity. TMPD oxidation by two isoforms of COX enzyme (COX-1 and COX-2) was examined in the presence of indicated concentrations of the crude extract. Reactions were initiated with arachidonic acid, and TMPD oxidation was monitored at 590 nm. Details of the assay conditions are described under *Materials and Methods.* Each plot represents the mean ± S.D. (triplicate determinations) of the relative activity to the control. *significantly different compared with the control group ($p < 0.05$).

TABLE 1

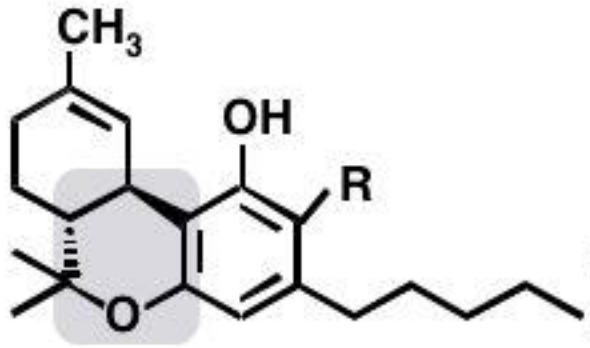
Comparison of IC_{50} values (μM) of various COX inhibitors[a]

Inhibitors	COX-1	COX-2	COX-1/COX-2 ratio[b]	Reference
CBDA	20	2.20	9.1	This work
Celecoxib	26.61	0.44	60.48	Tsai et al. (2006)
Diclofenac	0.06	0.22	0.27	Johnson et al. (1995)
Indomethacin	0.004	0.34	0.01	Tsai et al. (2006)

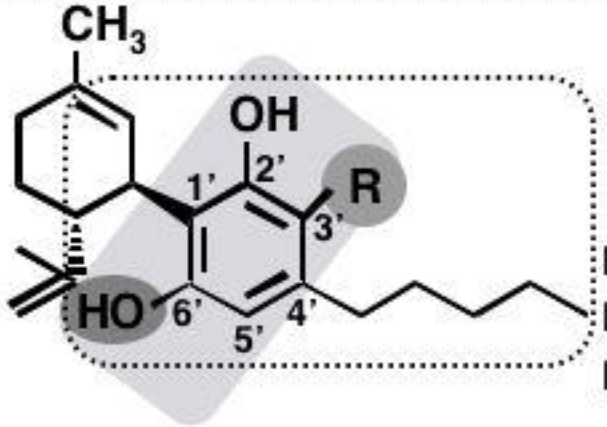
[a]The IC_{50} values for each of the inhibitors are cited from which the study is performed by using ovine COX-1 and COX-2 as enzyme sources (see *Materials and Methods*).

[b]The ratio of the IC_{50} values for COX-1 and COX-2 can be used as an indication of the COX-2 selectivity of inhibitors. A COX-1/COX-2 ratio of more than 1 indicates preferential COX-2 selectivity.

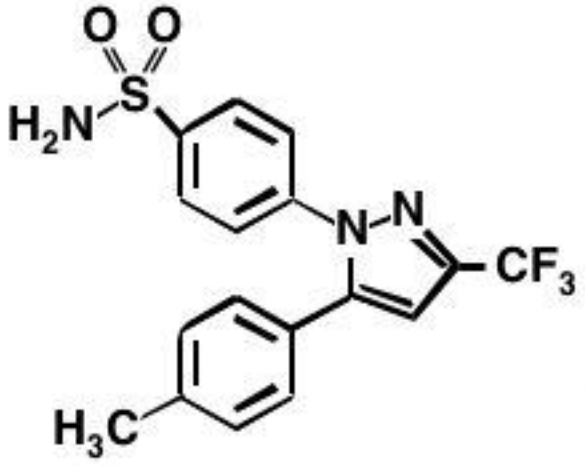
Fig. 1



R = H, Δ^9-THC
R = COOH, Δ^9-THCA



R = H, CBD
R = COOH, CBDA
R = $COOCH_3$, CBDA methyl ester



Celecoxib

Fig. 2

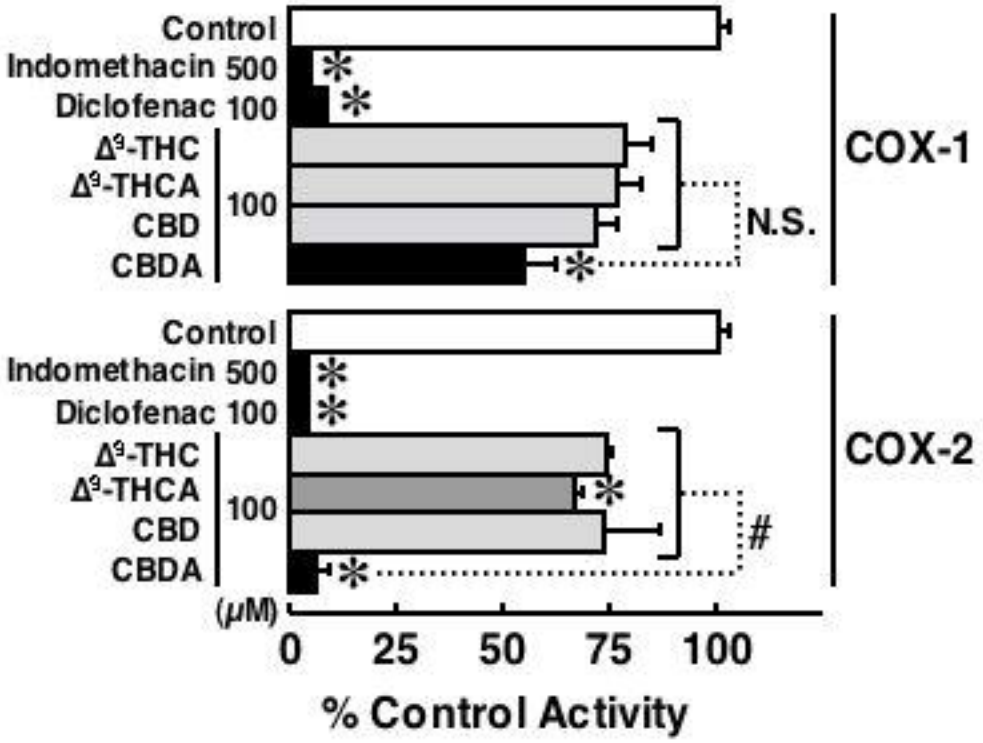

Control
Indomethacin 500
Diclofenac 100
Δ9-THC
Δ9-THCA
CBD
CBDA
100
N.S.
COX-1
Control
Indomethacin 500
Diclofenac 100
Δ9-THC
Δ9-THCA
CBD
CBDA
100
#
COX-2
(μM)
0
25
50
75
100
% Control Activity

Fig. 3

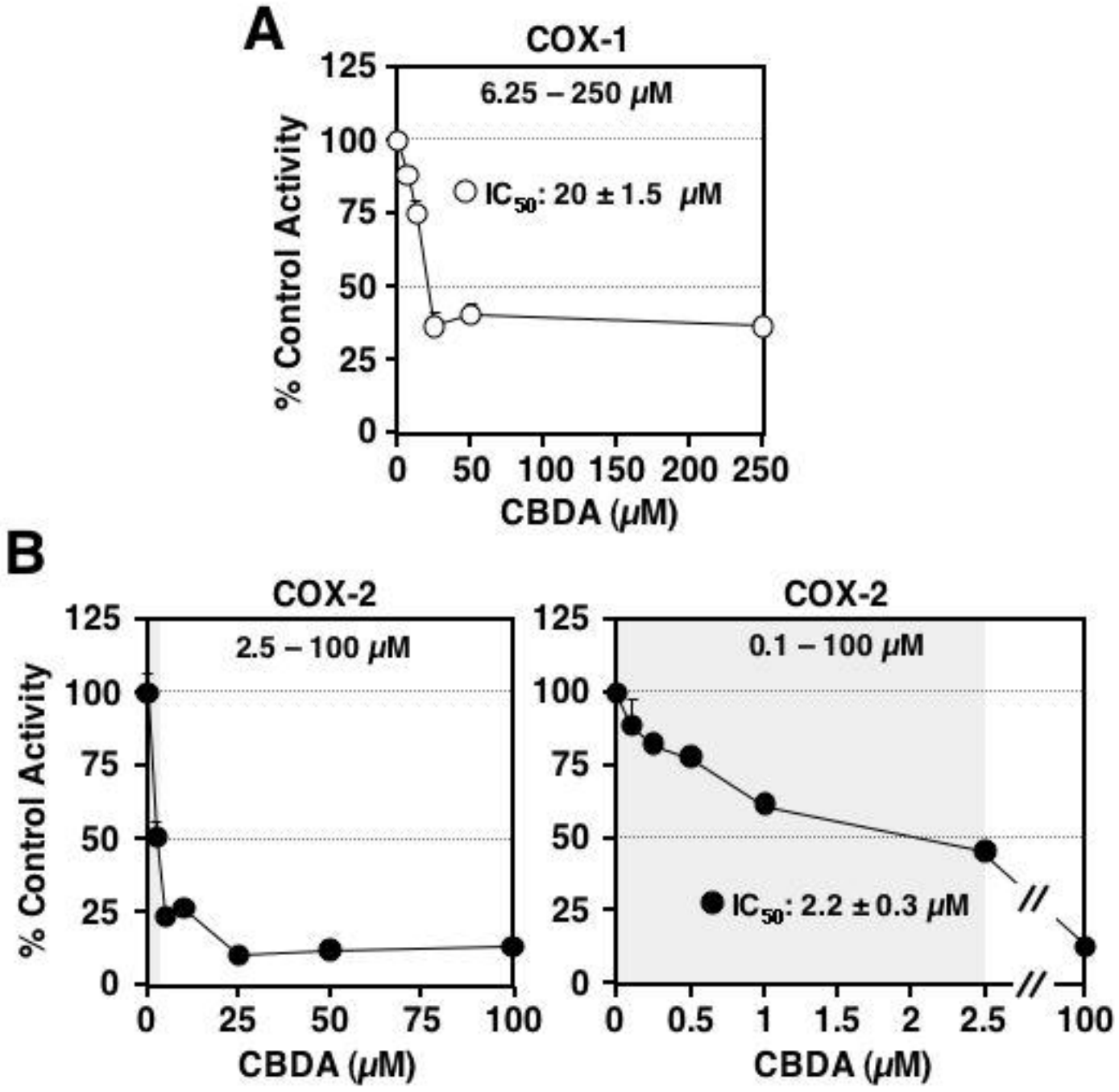

A
COX-1
6.25 – 250 μM
IC50: 20 ± 1.5 μM
% Control Activity
CBDA (μM)
B
COX-2
2.5 – 100 μM
COX-2
0.1 – 100 μM
IC50: 2.2 ± 0.3 μM
% Control Activity
CBDA (μM)
CBDA (μM)

Fig. 4

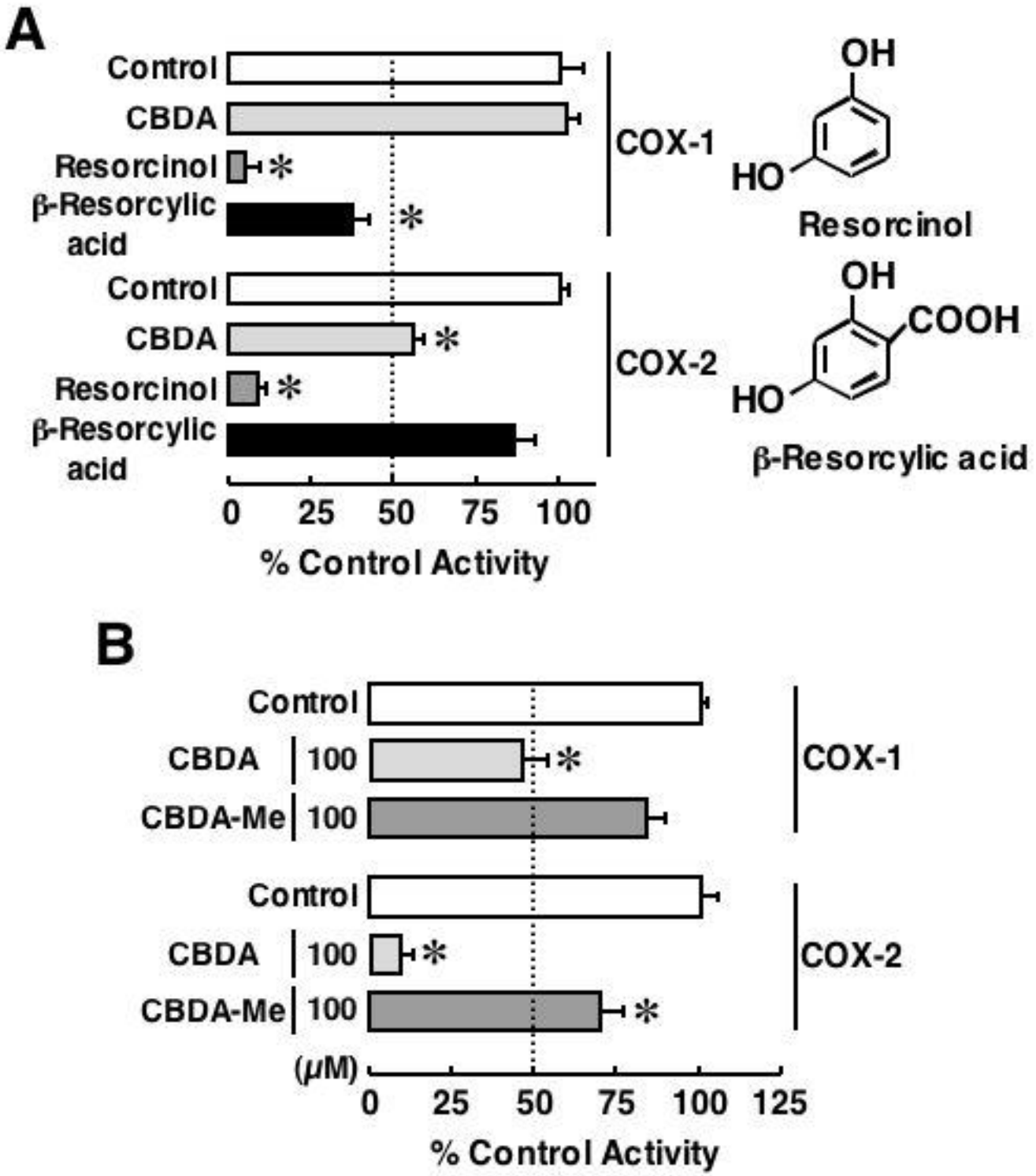
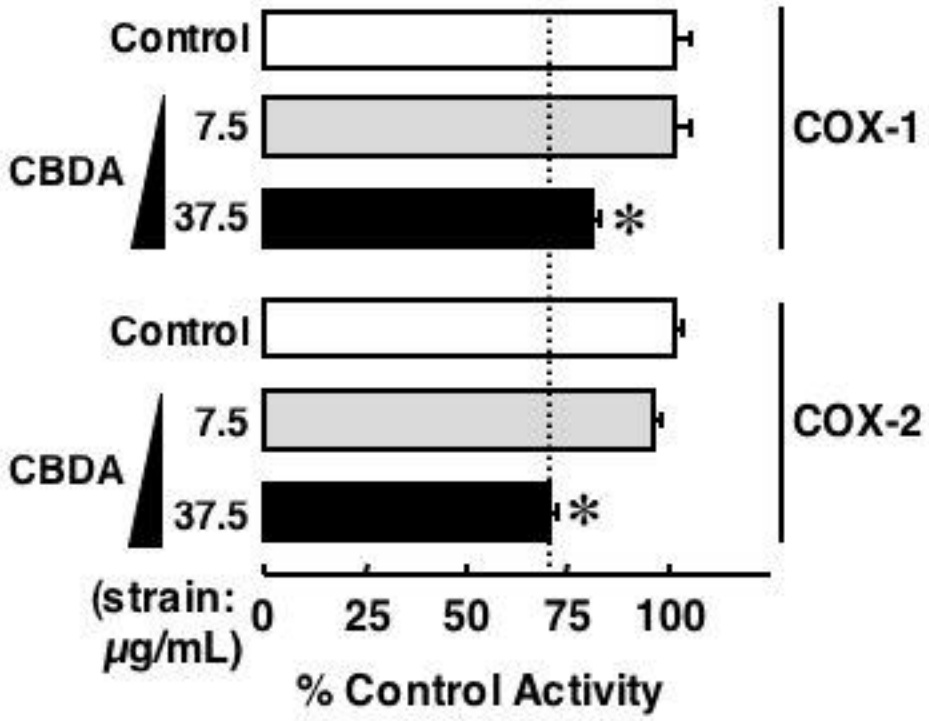

A
Control
CBDA
Resorcinol
β-Resorcylic acid
COX-1
Control
CBDA
Resorcinol
β-Resorcylic acid
COX-2
*
0 25 50 75 100
% Control Activity
OH
HO
Resorcinol
OH
COOH
HO
β-Resorcylic acid
B
Control
CBDA 100
CBDA-Me 100
COX-1
Control
CBDA 100
CBDA-Me 100
COX-2
(μM)
0 25 50 75 100 125
% Control Activity

Fig. 5


Control
7.5
CBDA
37.5
*
COX-1
Control
7.5
CBDA
37.5
*
COX-2
(strain: μg/mL)
0
25
50
75
100
% Control Activity