

Toxicomics Report

Down-regulation of cyclooxygenase-2 (COX-2) by cannabidiolic acid in human breast cancer cells

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ABSTRACT — Metastases are known to be responsible for approximately 90% of breast cancer-related deaths. Cyclooxygenase-2 (COX-2) is involved not only in inflammatory processes, but also in the metastasis of cancer cells; it is expressed in 40% of human invasive breast cancers. To comprehensively analyze the effects of cannabidiolic acid (CBDA), a selective COX-2 inhibitor found in the fiber-type cannabis plant (Takeda *et al.*, 2008), on COX-2 expression and the genes involved in metastasis, we performed a DNA microarray analysis of human breast cancer MDA-MB-231 cells, which are invasive breast cancer cells that express high levels of COX-2, treated with CBDA for 48 hr at 25 μ M. The results obtained revealed that COX-2 and Id-1, a positive regulator of breast cancer metastasis, were down-regulated (0.19-fold and 0.52-fold, respectively), while SHARP1 (or BHLHE41), a suppressor of breast cancer metastasis, was up-regulated (1.72-fold) and CHIP (or STUB1) was unaffected (1.03-fold). These changes were confirmed by real-time RT-PCR analyses. Taken together, the results obtained here demonstrated that i) CBDA had dual inhibitory effects on COX-2 through down-regulation and enzyme inhibition, and ii) CBDA may possess the ability to suppress genes that are positively involved in the metastasis of cancer cells *in vitro*.

Key words: Cannabidiolic acid, Cyclooxygenase-2, Human breast cancer cells, Fiber-type cannabis plant, Metastasis

INTRODUCTION

Cannabidiol (CBD), a major constituent of the fiber-type cannabis plant, exhibits a wide range of biological activities, such as anti-cancer cell proliferation, anti-cerebral infarction, and the inhibition of 15-lipoxygenase (Mishima *et al.*, 2005; Izzo *et al.*, 2009; Takeda *et al.*, 2009; Caffarel *et al.*, 2012; Takeda, 2013). In fresh plant materials, most CBD exists in its acid form, cannabidiolic acid (CBDA, Fig. 1A) (Yamauchi *et al.*, 1967; Turner *et al.*, 1980; Taura *et al.*, 2007). The specific use of the acidic cannabinoid as an active pharmaceutical ingredient has not yet been achieved because CBDA is recognized as the

pharmacologically inactive form (Yamauchi *et al.*, 1967; Razdan, 1986; Burstein, 1999). However, recent studies including ours demonstrated that, in addition to CBD, CBDA by itself exhibits biological actions, such as antibacterial effects (Appendino *et al.*, 2008), the inhibition of cyclooxygenase-2 (COX-2) (Takeda *et al.*, 2008), and anti-nausea/emetic effects (Bolognini *et al.*, 2013; Rock *et al.*, 2013).

Approximately 90% of breast cancer-related deaths have been attributed to metastasis to bones and the lungs (Hanahan and Weinberg, 2011). COX-2 is expressed in approximately 40% of human invasive breast cancers (Singh *et al.*, 2007; Holmes *et al.*, 2011), and may

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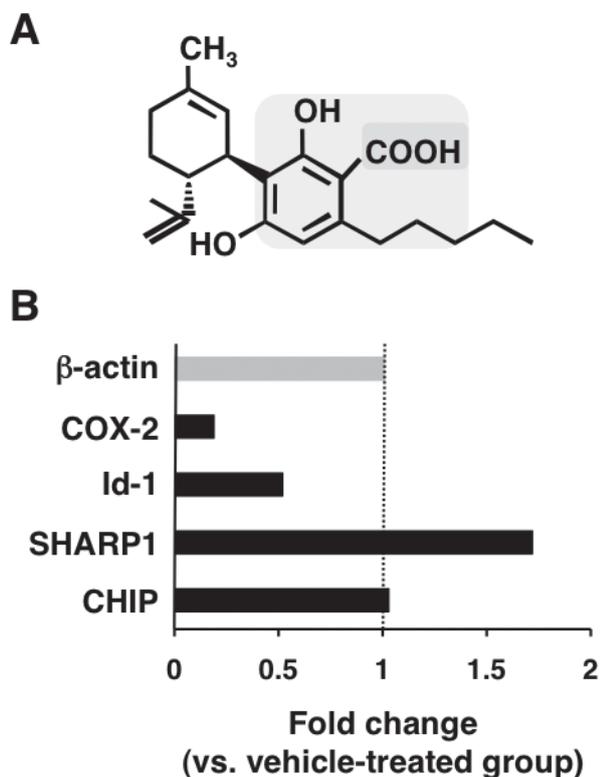


Fig. 1. Effects of CBDA on the expression of COX-2 and CHIP in MDA-MB-231 cells. (A) The chemical structure of CBDA is shown. The salicylic acid moiety in the structure was indicated with a gray inclusion. CBD can be formed from CBDA by decarboxylation ($-\text{COOH}$). (B) The results of the DNA microarray analysis. Data are expressed as fold change vs. vehicle-treated groups. MDA-MB-231 cells were treated with vehicle or 25 μM CBDA for 48 hr, followed by the isolation of total RNA. Details of the microarray conditions are described in the Materials and Methods.

be actively involved in breast cancer metastasis to bones and the lungs (Yoshinaka *et al.*, 2006; Singh *et al.*, 2007). Thus, one strategy to reduce the risk of breast cancer including metastasis involves COX-2 inhibitors.

Previous studies reported that COX-2 gene expression exhibited a significant linear relationship with tumor cell density (Brueggemeier *et al.*, 1999; Davies *et al.*, 2002). This findings prompted us to investigate the effects of CBDA on COX-2 expression in MDA-MB-231 breast cancer cells, which were established as a breast cancer model for preclinical studies because they are highly aggressive, both *in vitro* and *in vivo* (Price *et al.*, 1990). In the present study, we also analyzed the effects of CBDA on the genes involved in metastasis by DNA microarray

analyses.

MATERIALS AND METHODS

Materials and cell culture

CBDA (purity: 96.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DuP-697 (purity: > 96%) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other reagents were of analytical grade, commercially available, and used without further purification. Cell culture (human breast cancer MDA-MB-231 cells) conditions and methods were based on procedures described previously (Takeda *et al.*, 2011, 2013a). Briefly, the MDA-MB-231 cell line (obtained from the American Type Culture Collection, Rockville, MD, USA) was routinely grown in phenol red-containing minimum essential medium alpha (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES, 5% fetal bovine serum, 100 U/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin at 37°C in a 5% CO_2 -95% air-humidified incubator. Prior to the 24-hr chemical treatments, the medium was changed to phenol red-free minimum essential medium alpha (Invitrogen) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum, 100 U/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin. Cells were then re-seeded into 6-well plates at different densities; 30 $\times 10^5$ cells (31,250 cells/cm²), 40 $\times 10^5$ cells (41,667 cells/cm²), or 50 $\times 10^5$ cells (52,083 cells/cm²)/well. CBDA and DuP-697 were prepared in ethanol and dimethylsulfoxide (DMSO), respectively. Control incubations contained equivalent additions of ethanol or DMSO.

Preparation of total RNA and DNA microarray analyses

Total RNA was collected from two COX-2 inhibitors (25 μM CBDA or 25 μM DuP-697) or vehicle-treated MDA-MB-231 cells (30 $\times 10^5$ cells/well) 48 hr after exposure using the RNeasy kit (Qiagen, Inc., Hilden, Germany), and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). The specific gene expression pattern in MDA-MB-231 cells was examined by DNA microarray analysis and compared with that in vehicle-controls. Total RNA was extracted from both cell types, and the synthesis of complementary DNA (cDNA) and cRNA labeling were conducted using a Low RNA Fluorescent Linear Amplification kit (Agilent, Palo Alto, CA, USA). Overall changes in gene expression were evaluated using two-color microarray-based gene expression analysis (Hwang *et al.*, 2011; Takeda *et al.*, 2011, 2013b; Toyama *et al.*, 2011). Labeled cRNA (Cy3 to control, Cy5 to

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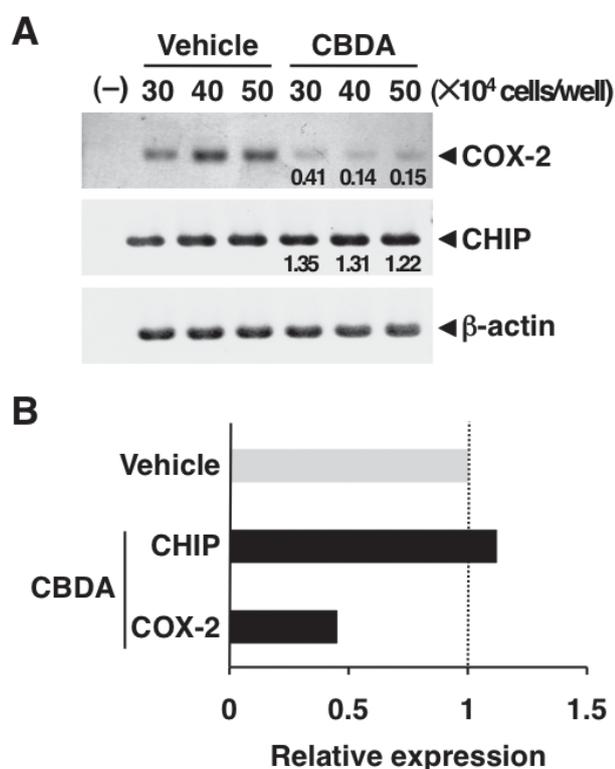


Fig. 2. CBDA-mediated down-regulation of COX-2. (A) RT-PCR analyses of COX-2 and CHIP levels in MDA-MB-231 cells seeded at different densities; 30, 40, or 50 $\times 10^5$ cells/well 48 hr after the CBDA (25 μ M) or vehicle treatment. β -Actin was used as an internal loading control. Band intensities were quantified by densitometry (ImageJ 1.46r software) and normalized to β -actin levels. The normalized values were converted to fold changes relative to the respective vehicle-treated controls. When mock reverse transcription reactions were performed with the omission of reverse transcriptase (indicated as -), no signal was detected in any of the samples. (B) Real-time RT-PCR analyses of COX-2 and CHIP levels in MDA-MB-231 cells seeded at a density of 30 $\times 10^5$ cells/well 48 hr after the CBDA (25 μ M) or vehicle treatment. The values were converted to fold changes relative to vehicle-treated controls.

CBDA) was hybridized to human oligo DNA microarray slides (Agilent) that carried spots for human genes. Specific hybridization was analyzed using a Microarray scanner (Agilent) and evaluated as a scatter-plot graph for gene expression. Hokkaido System Science (Sapporo, Japan) provided assistance with the experiments.

Analysis of reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR

Total RNA was prepared from MDA-MB-231 cells

using the RNeasy kit (Qiagen, Inc.) and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). The following synthesis of cDNA, RT, and PCR were performed using the SuperScript One-Step RT-PCR System with Platinum *Taq* polymerase (Invitrogen). Primers for the PCR of β -actin, CHIP, and COX-2 were taken from previous studies (Wu *et al.*, 2003; Dikshit and Jana, 2007; Takeda *et al.*, 2011). PCR was performed under conditions that produced template quantity-dependent amplification. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-acetate EDTA (ethylenediamine-*N,N,N',N'*-tetraacetic acid) buffer and stained with ethidium bromide. When the RT reaction was omitted (indicated as -), no signal was detected in any of the samples (see Fig. 2A). β -Actin was used as an internal control for RT-PCR. The quantification of band intensities was performed using ImageJ 1.46r software (<http://imagej.nih.gov/ij/>). In the real-time RT-PCR analysis of COX-2 and CHIP, cDNA was prepared via RT of total RNA using the ReverTra Ace[®] qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). A real-time quantitative RT-PCR assay was performed with FastStart Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) and the LightCycler Nano (Roche Diagnostics, Mannheim, Germany). Primers for the PCR of β -actin, COX-2, and CHIP were taken from previous studies (Wu *et al.*, 2003; Shigematsu *et al.*, 2009; Roelofs *et al.*, 2014). The reaction conditions for COX-2 were 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, at 58°C for 10 sec, and 72°C for 15 sec, and for CHIP were 95°C for 10 min, followed by 45 cycles at 95°C for 10 sec, at 60°C for 10 sec, and 72°C for 15 sec. COX-2 and CHIP mRNA levels were normalized to the corresponding β -actin mRNA levels.

RESULTS AND DISCUSSION

The anti-proliferative potential of CBDA (Fig. 1A) on MDA-MB-231 cell growth was shown to be markedly less than that of CBD (IC₅₀ values of CBD and CBDA = 1~10 μ M and > 25 μ M, respectively) (Ligresti *et al.*, 2006; McAllister *et al.*, 2007; Shrivastava *et al.*, 2011). In this study, exposure of MDA-MB-231 cells to CBDA (25 μ M) for 48 hr did not affect cell viability (data not shown) (Takeda *et al.*, 2012). Under the condition (25 μ M CBDA for 48 hr), we performed DNA microarray analysis to investigate genes regulated by CBDA in MDA-MB-231 cells (cell density: 30 $\times 10^5$ cells/well). Among 19,596 genes, CBDA increased the expression of 449 genes (> 2-fold) and decreased the expression 639 genes (< 0.5-fold) in MDA-MB-231 cells. We focused on four

genes involved in the metastasis of breast cancer: COX-2, Id-1, CHIP (also known as STUB1), and SHARP1 (also known as BHLHE41). The expression of COX-2 and Id-1 was down-regulated 0.19-fold and 0.52-fold, respectively, by CBDA, while SHARP1, a suppressor of breast cancer metastasis (Montagner *et al.*, 2012) was up-regulated (1.72-fold). However, CHIP, which acts as a suppressor of breast cancer metastasis (Kajiro *et al.*, 2009), was unaffected (1.03-fold) by the cannabinoid (Fig. 1B). A previous study reported that the Id-1 gene, a positive regulator of breast cancer metastasis/progression (Fong *et al.*, 2003), was down-regulated by CBD (McAllister *et al.*, 2007); therefore, CBDA, the precursor of CBD (*see* Fig. 1A), may use a similar mechanism(s) to CBD in order to suppress the expression of Id-1.

COX-2 expression was previously suggested to correlate with tumor cell density (Brueggemeier *et al.*, 1999; Davies *et al.*, 2002). We focused on COX-2 and CHIP, and examined the effects of breast cancer cell density on their expression. As shown in Fig. 2A, COX-2 expression increased slightly as a function of the density of cells ranging from 30 to 50 x 10⁵ cells/well, whereas the addition of CBDA to these cells down-regulated COX-2 expression. In contrast, CHIP expression was not markedly modulated by either CBDA or cell density (*see* also Fig. 2B, 1.12-fold). The down-regulation of COX-2 by CBDA was also demonstrated using real-time RT-PCR analyses (0.45-fold) (Fig. 2B). Although the inability of CBDA to modulate the expression of CHIP in MDA-MB-231 cells was revealed, CBDA may target gene(s) whose expression can be affected by cancer cell density.

Although we demonstrated for the first time that CBDA was a potent inhibitor of COX-2 (IC₅₀ = 2.2 μM) purified from sheep placental cotyledons, which are a general enzyme source for screening (Cayman Chemical Company) (Takeda *et al.*, 2008), a contradictory phenomenon, in which CBDA exhibited a very weak inhibitory potential on COX-2, was recently reported (Ruhaak *et al.*, 2011). Since this research group and we used the same enzyme source, this discrepancy may have been caused by differences in the experimental conditions or purity of CBDA. In the present study, we could not definitively identify the molecular mechanism(s) underlying the CBDA-mediated inhibition of COX-2 expression; however, previous studies reported that sodium salicylate inhibited transcription of the COX-2 gene, which was driven by the transcription factors, activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) (Kopp and Ghosh, 1994; Dong *et al.*, 1997; Xu *et al.*, 1999). Since CBDA contains a salicylic acid moiety in its structure (*see* Fig. 1A, the moiety is highlighted with a gray inclusion), the possible involvement

of this moiety is implicated. Furthermore, we investigated the effect of DuP-697, an established COX-2 inhibitor (IC₅₀ = 0.05 μM) (Cayman Chemical), on the expression of Id-1, SHARP1, and CHIP genes using real-time RT-PCR analysis. As in the case of CBDA, 25 μM DuP-697 exhibited up-regulation of SHARP1 (1.48-fold) and no remarkable change in CHIP (1.20-fold); however, Id-1 expression, which can be down-regulated by CBDA, was largely unchanged by the inhibitor (0.82-fold) (*see* Fig. 1B). Thus, it is suggested that stimulation of SHARP1 expression results from COX-2 inhibition, but reduction in expression of Id-1 is caused by CBDA's property as mentioned above.

Taken together, the results of the present study suggest that COX-2 inhibitors that can down-regulate the enzyme may also be useful in inhibiting it, which may abrogate metastasis. Further studies are needed to demonstrate the biological effects of CBDA *in vivo*.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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