Cannabidiol hydroxyquinone-induced apoptosis of splenocytes is mediated predominantly by thiol depletion

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Cannabidiol, the major nonpsychotropic phytocannabinoid, has been recently demonstrated to induce apoptosis in primary lymphocytes via an oxidative stress-dependent mechanism. Cannabidiol can be converted by microsomal enzymes to the hydroxyquinone metabolite HU-331 that forms adducts with glutathione. The present study tested the hypothesis that HU-331 could cause apoptosis via the depletion of thiols in splenocytes. Our results showed that HU-331 treatment significantly enhanced splenocyte apoptosis in a time- and concentration-dependent manner. Concordantly, a gradual diminishment in the cellular thiols and glutathione was detected in HU-331-treated splenocytes. The apoptosis and thiol diminishment induced by HU-331 were abrogated in the presence of thiol antioxidants, including N-acetyl-l-cysteine and N-(2-mercaptobenzyl) glycine, whereas the non-thiol antioxidants catalase and pyruvate were ineffective. In comparison, both thiol and non-thiol antioxidants were capable of attenuating H2O2-induced thiol diminishment and reactive oxygen species generation in splenocytes. Collectively, these results suggest that HU-331 might be an active metabolite of cannabidiol potentially contributing to the induction of apoptosis in splenocytes, and that the apoptosis is primarily mediated by the loss of cellular thiols.

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1. Introduction

Cannabidiol (CBD) is one of the major cannabinoid compounds derived from the plant Cannabis sativa, also known as marijuana (Lerner, 1963). One of the most distinctive features of CBD is the lack of psychotropic activity due to its low affinity to the known cannabinoid receptors CB1 and CB2 (Howlett, 1995). This property renders CBD an attractive candidate as a potential therapeutic agent currently under intensive investigation (Costa et al., 2007; Mechoulam and Hanus, 2002; Strasser et al., 2006; Zuardi et al., 2006). Increasing evidence indicates that CBD exhibits a versatile spectrum of pharmacological effects, including neuroprotection, antipsychosis, anticancer, anti-inflammation and immune modulation (Mechoulam and Hanus, 2002). Many studies demonstrate that CBD produces a broad spectrum of immunomodulatory effects on the functionality of various immune cells, such as lymphocytes (Costa et al., 2007; Jan et al., 2007; Rajesh et al., 2007; Weiss et al., 2006). For example, we have recently shown that both transformed and primary lymphocytes were sensitive to the induction of apoptosis by CBD (Lee et al., 2008; Wu et al., 2008). These results suggest on one hand the potential antitumor, and on the other hand the immunotoxic effect of CBD. To date, the precise mechanism underlying the pro-apoptotic effect of CBD in lymphocytes remains largely unclear. Several studies, including ours, suggest a pivotal role of oxidative stress in the pro-apoptotic effect of CBD, as evidenced by the increased production of reactive oxygen species (ROS) and the depletion of cellular thiols in both transformed and non-transformed leukocytes exposed to CBD (Lee et al., 2008; Massi et al., 2006; McKallip et al., 2006; Wu et al., 2008).

The reduction–oxidation (redox) homeostasis of the cell plays a critical role in the regulation of many cellular functions, such as proliferation and apoptosis (Mates et al., 2008). Interference with the redox balance is known to be a key mechanism involved in the induction of apoptosis by various cytotoxic agents (Davis et al., 2001; Huang et al., 2003). The cellular thiols, in particular glutathione and thioredoxin, are the major intracellular antioxidant buffers against oxidative stress. In addition, thiols participate in various cellular functions, such as proliferation and apoptosis by modulating the activity of many regulatory components of the apoptotic process, including signaling molecules, transcription factors and cysteine proteases (Biswas et al., 2006; Circu and Aw, 2008). The capability of glutathione and its precursor N-acetyl-l-cysteine (NAC) to rescue cells from apoptotic death has been well documented (Kim and Nel, 2005; Rosati et al., 2004). Of relevant to the present study, we previously reported that CBD-induced apoptosis in splenic lymphocytes was abrogated in the presence of NAC...
Thiol antioxidants, but not non-thiol antioxidants, attenuated HU-331-mediated thiol diminishment in splenocytes (Wu et al., 2008). Likewise, HU-331-mediated cytotoxicity in Jurkat T cells was significantly attenuated by NAC pretreatment (Kogan et al., 2007b).

CBD has been shown to be metabolized by hepatic microsomes to form a quinone metabolite, cannabidiol hydroxyquinone (HU-331), which has been suggested as a reactive metabolite of CBD based on its reactivity with glutathione and cytochrome P450 3A11 (Bornheim and Grillo, 1998). Notably, HU-331 exhibited anti-proliferative and pro-apoptotic activities; the growth of several transformed cells, including Jurkat T cells, MCF-7 breast cancer and HT-29 colon cancer cells was suppressed by HU-331 (Kogan et al., 2004). In vivo study further showed that HU-331 inhibited the growth of HT-29 tumor xenografts in nude mice, with a greater potency and less cardiotoxicity than doxorubicin, an anticancer quinone commonly used in clinical (Kogan et al., 2004, 2007a). In addition to the direct cytotoxic effect on tumor cell growth, HU-331 possessed anti-angiogenetic property. HU-331 suppressed the angiogenesis of aortic rings ex vivo and HT-29 tumor implants in vivo via the induction of apoptosis of vascular endothelial cells (Kogan et al., 2006). However, the mechanism of HU-331-mediated apoptosis remains obscure.

Antitumor quinones, such as anthracenes, are known to induce apoptosis in both immortalized and normal leukocytes (Friesen et al., 1999; Gouaze et al., 2001; Kalivendi et al., 2005; L’Ecuyer et al., 2004). As aforementioned, CBD can be converted to HU-331, a cannabinoid quinone possessing cytotoxic activity in various transformed lines and primary endothelial cells, we hypothesized that HU-331 might be an active metabolite of CBD, which produce apoptotic effect in primary lymphocytes. We report here that HU-331 markedly induced splenocyte apoptosis, which is mediated primarily by the diminishment of cellular thiols.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. HU-331 was purchased from Cayman Chemical (Ann Arbor, MI). The solvent of HU-331, methyl acetate, was evaporated by a gentle stream of nitrogen and HU-331 was re-dissolved in absolute ethanol to a concentration of 4 mM. This stock solution of HU-331 was aliquoted into amber vials, purged with nitrogen, and stored at −20°C. Each vial of the HU-331 stock solution was used once and further diluted to the desired concentrations with Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT). Fetal bovine serum (FBS) and cell culture reagents were purchased from Gibco BRL (Gaithersburg, MD).

2.2. Animals and cell cultures

Male BALB/c mice, 4–5 weeks of age were purchased from the Animal Breeding Center of the National Taiwan University Hospital (Taipei, Taiwan). On arrival, mice were randomized, transferred to plastic cages containing a saw-dust bedding (five mice per cage) and quarantined at least for 1 week. The animal room was maintained with a temperature of 24 ± 2°C and a relative humidity of 60 ± 20%, with a 12-h light/dark cycle. Mice were fed standard laboratory food and water ad libitum. Their spleens were isolated aseptically and made into single cell suspensions as described previously (Kaminski et al., 1994). The erythrocytes in splenocyte cultures were lysed using a hypotonic buffer (150 mM NH4 Cl, 10 mM KHCO3 and 100 mM Na2EDTA; pH 7.2). The splenocytes were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% heat inactivated FBS (referred to as complete RPMI). In all cases, the cells were cultured at 37°C in a 5% CO2 incubator.

2.3. Cell cycle analysis

Cell cycle distribution of splenocytes was measured by flow cytometry using propidium iodide (PI) staining as previously described (Wu et al., 2008). Briefly, the cells (5 × 10⁶ cells/ml) were either left untreated (naïve; NA), or treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 1–12 h. At the end of incubation, the cells were fixed with 70% ethanol and subjected to cell cycle analysis. The single cell fluorescence of 5000 cells for each sample was measured using a flow cytometer (BD FACs Calibur, San Jose, CA). The PI emission was detected in the FL2 channel using emission filter of 575 nm. The apoptotic cells were defined as those cells in sub-G0/G1 phase with hypodiploid DNA content (Nicoletti et al., 1991). The data were analyzed using the software Flowjo 5.7.

2.4. Terminal dUTP nick-end labeling (TUNEL) assay

Splenocytes (5 × 10⁶ cells/ml) were treated with HU-331, and fixed with ethanol as described above. The DNA strand breaks of apoptotic cells were measured using a commercial TUNEL assay kit (Roche Diagnostics GmbH, Penzberg, Germany) following the manufacturer’s instruction with some modifications. Briefly, fixed splenocytes were stained with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP for 1 h at 37°C in the dark. Cells were then washed twice with PBS and the single cell fluorescence of 5000 cells for each sample was measured using a flow cytometer at emission of 525 nm. The data were analyzed using the software Flowjo 5.7. 2.5. Detection of intracellular thiols using flow cytometry

Splenocytes were either treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 1–12 h as described earlier, or treated with H2O2 (50 μM) for 12 h. After the treatment, the cells were washed, incubated with 5-chloromethylfluorescein diacetate (CMF-DA; 20 μM) for 25 min at 37°C in the dark and then analyzed by flow cytometry. The single cell fluorescence of 10,000 cells for each sample was measured using a flow cytometer at emission of 525 nm. The data were analyzed using the software Flowjo 5.7.

2.6. Measurement of intracellular reactive oxygen species (ROS)

Splenocytes were preloaded with dichlorofluorescin diacetate (DCF-DA; 20 μM) for 30 min at 37°C. After washing, the splenocytes (5 × 10⁶ cells/ml) loaded with DCF-DA were cultured in triplicate in a 24-well plate (0.5 ml/well) and treated with H2O2 (50 μM) for 2 h. After washing with PBS the single cell fluorescence of 10,000 cells for each sample was measured using a flow cytometer at emission of 525 nm. The data were analyzed using the software Flowjo 5.7.

2.7. Enzymatic measurement of intracellular glutathione

Splenocytes were either left untreated (NA) or treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 12 h. After treatment, the cells were washed and the intracellular levels of glutathione were measured spectrophotometrically with a commercial Glutathione Assay Kit (Cayman Chemical Company, Ann Arbor, MI). Briefly, cells were homogenized by sonication in an ice-cold buffer solution (0.2 M 2-(N-morpholino)ethanesulfonic acid, 50 mM phosphate and 1 mM EDTA, pH 6.0). After centrifugation, the supernatants were collected and the glutathione level was quantified following the supplier’s instructions.

2.8. Detection of caspase-8 activation

The activation of caspase-8 was measured by flow cytometry using specific cell-permeable substrates of caspase-8 (FAM-LETD-FMK; CaspaTag Assay Kit, CHEMICON International Inc., Temecula, CA). Briefly, splenocytes were treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) in the absence or presence of NAC

| Table 1 |
| Thiol antioxidants, but not non-thiol antioxidants, attenuated HU-331-mediated thiol diminishment in splenocytes. |
| Control | NAC (mM) | MPG (mM) | Catalase (400 U/mL) | Pyruvate (10 mM) |
| 0.001 | 0.01 | 1 | 0.001 | 0.01 | 1 |
| VH | 548 ± 20 | 477 ± 54 | 544 ± 22 | 599 ± 12 | 586 ± 7 | 561 ± 16 | 543 ± 10 | 560 ± 15 | 570 ± 17 | 560 ± 6 | 585 ± 84 |
| HU-331 | 176 ± 13 | 167 ± 4 | 361 ± 5 | 556 ± 14 | 587 ± 18 | 154 ± 5 | 367 ± 8 | 546 ± 22 | 589 ± 21 | 169 ± 1 | 180 ± 7 |

* Splenocytes (5 × 10⁶ cells/ml) were treated with HU-331 (1 μM) and/or VH (0.05% ethanol) for 12 h in the absence (control group) or presence of NAC (0.001–1 mM), MPG (0.001–1 mM), catalase (400 U/mL) or pyruvate (10 mM). The cells were then incubated with CMF-DA (20 μM) for 25 min, and the CMF fluorescence was measured by flow cytometry. Data (mean fluorescence of CMF) are expressed as the mean ± SE of triplicate samples per group.

p < 0.05 as compared to the control group treated with HU-331. Results are a representative of three independent experiments.

Table 2

| Thiol antioxidants, but not non-thiol antioxidants, attenuated HU-331-mediated apoptosis in splenocytes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control | NAC (0.001–1 mM) | MPG (0.001–1 mM) | Catalase (400 U/mL) | Pyruvate (10 mM) |
| VH | 29.8 ± 0.9 | 30.4 ± 0.9 | 23.0 ± 1.5 | 21.9 ± 0.4 |
| HU-331 (0.5 M) | 27.4 ± 0.6 | 27.4 ± 0.6 | 27.4 ± 0.6 | 27.4 ± 0.6 |
| HU-331 (1 M) | 26.8 ± 0.9 | 26.8 ± 0.9 | 26.8 ± 0.9 | 26.8 ± 0.9 |

(1 mM) for 12 h. After washing with PBS, the cells were incubated with FAM-LETD-FMK for 1 h at 37 °C in the dark, and then washed again. The single cell fluorescence of 5000 cells for each sample was measured using a flow cytometer at emission of 525 nm. The data were analyzed using the software FlowJo 5.7.

2.9. Statistical analysis

The mean ± standard error (SE) was determined for each treatment group in the individual experiments. Dunnett’s two-tailed t-test was used to compare HU-331-treated groups to the control group. For experiments with antioxidants pretreatment (Tables 1 and 2 and Fig. 3), the data were evaluated by two-way analysis of variance, and Duncan’s multiple range test using the Statistical Analysis System (SAS Institute Inc., Cary, NC). p-Value <0.05 was defined as statistical significance.

3. Results

3.1. HU-331 enhanced apoptosis in splenocytes

We first examined the effect of HU-331 on the apoptosis of splenocytes. Freshly isolated splenocytes from naive mice were used throughout the studies. Primary splenocytes in culture gradually underwent spontaneous apoptosis; the apoptotic rates were approximately 10 and 25% at 1 and 12 h, respectively (data not shown). Splenocytes were either left untreated (naïve; NA), or exposed to HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 1–12 h, and the hypodiploid (sub-G0/G1) apoptosis was quantified by cell cycle analysis. As shown in Fig. 1A, exposure of cells to HU-331 (0.5–2 μM) for 12 h markedly enhanced apoptosis in a concentration-dependent manner with the 2 μM group reaching 80% of apoptotic rate. Concentrations of HU-331 greater than 2 μM caused both necrosis and apoptosis (data not shown). The effect of HU-331 (1 μM) was also time-dependent between 3 and 12 h (Fig. 1B). The apoptosis was confirmed by the results of TUNEL staining, which showed a marked increase in the proportion of DNA strand breaks in HU-331-treated splenocytes (Fig. 1C and D). Representative histograms of the TUNEL staining of VH- and HU-331 (1 μM)-treated cells are illustrated in Fig. 1E. Both cell cycle analysis and TUNEL staining demonstrated a similar magnitude and kinetics of HU-331-mediated apoptotic effect (Fig. 1).

3.2. HU-331 induced diminishment of thiols and glutathione in splenocytes

Previous reports have shown that HU-331 can form adducts with glutathione. We therefore examined whether HU-331 influenced the cellular thiols in splenocytes. Flow cytometric analysis using CMF-DA demonstrated that HU-331 treatment induced a marked diminishment in the cellular thiols in a concentration (0.5–2 μM)- and time (3–12 h)-dependent manner (Fig. 2A and B, respectively). We further measured the intracellular glutathione using an enzymatic assay, and the results confirmed that the cellular glutathione levels were markedly attenuated by HU-331 (0.5–2 μM) treatment for 12 h (Fig. 2C).

3.3. HU-331-mediated thiol diminishment and apoptosis in splenocytes were abrogated by thiol antioxidants, but not non-thiol antioxidants

To investigate the involvement of thiols in the apoptotic effect of HU-331, two thiol antioxidants, including N-acetyl-L-cysteine (NAC) and N-(2-mercapto-2-propionyl) glycine (MPG) were employed. Splenocytes were pretreated with NAC or MPG (0.001–1 mM of each) followed by HU-331 (1 μM) and/or VH (0.05% ethanol) treatment for 12 h, and the level of cellular thiols, and the apoptosis were measured. The presence of NAC or MPG (0.01–1 mM), in a concentration-dependent manner, significantly and gradually restored the diminished thiols in HU-331-treated cells; both NAC and MPG at concentrations ≥0.1 mM almost
Fig. 1. HU-331 enhanced apoptosis in murine splenocytes. (A and C) Splenocytes (5 × 10^6 cells/mL) were either left untreated (NA) or treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 12 h. (B and D) Splenocytes were treated with HU-331 (1 μM) and/or VH (0.05% ethanol) for 1–12 h. The apoptosis was measured using cell cycle analysis (A and B) or the TUNEL assay (C–E) as described in Section 2. Data are expressed as the mean ± SE of triplicate samples per group. *p < 0.05 as compared with the matched VH group. (E) Representative histograms of TUNEL analyses performed after 12-h exposure to HU-331 (1 μM) and/or VH were shown. Results are a representative of three independent experiments.

completely reversed the HU-331 effect (Table 1). Concordantly, HU-331-enhanced apoptosis was remarkably attenuated by the 2 thiol antioxidants (Table 2). In contrast to the effectiveness of the thiol antioxidants, non-thiol antioxidants including pyruvate (10 mM) and catalase (400 U/mL) were ineffective in reversing the HU-331 effects (Tables 1 and 2). We further conducted confirmatory studies to ensure the antioxidative activity of the employed antioxidants. Our results showed that both the thiol and non-thiol antioxidants were effective against H2O2 (50 μM)-induced thiol diminishment and ROS generation (Fig. 3A and B, respectively).

3.4. HU-331 activated caspase-8 in splenocytes, which was abrogated by NAC

We previously reported that CBD-mediated apoptosis was closely associated with an activation of caspase-8 in splenocytes (Wu et al., 2008). Therefore, the effect of HU-331 on the activation of caspase-8 in splenocytes was measured to further compare the profile of the pro-apoptotic property between HU-331 and CBD. Treatment of splenocytes with HU-331 (0.5–2 μM) markedly activated caspase-8 in a concentration-dependent manner (Fig. 4A and B). In addition, NAC was used to investigate the relationship between thiol diminishment and the caspase-8 activation induced by HU-331. The results showed that the activation of caspase-8 was abrogated in the presence of NAC (1 mM; Fig. 4A).

4. Discussion

Although the pro-apoptotic effect of CBD in lymphocytes has been well documented, its underlying mechanism is poorly understood. The present study aimed to investigate potential underlying mechanisms for CBD-mediating apoptosis in primary lymphocytes. Our data showed that HU-331 remarkably induced apoptosis in splenocytes, as evidenced by the increased hypodiploidity and DNA strand breaks in HU-331-treated cells (Fig. 1). The effective concentrations to induce splenocyte apoptosis by HU-331 (0.5–2 μM) are slightly less than that by CBD (4–8 μM) demonstrated in our previous report (Wu et al., 2008), indicating a greater potency for HU-331
HU-331 diminished the intracellular level of total thiols and glutathione (GSH) in splenocytes. (A) Splenocytes were either left untreated (NA) or treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 12 h. (B) Splenocytes were treated with HU-331 (1 μM) and/or VH (0.05% ethanol) for 1–12 h. The cells were then incubated with CMF-DA (20 μM) for 25 min, and the CMF fluorescence was measured by flow cytometry. Data are expressed as the mean ± SE of triplicate samples per group. *p < 0.05 as compared with the matched VH group. Results are a representative of three independent experiments. (C) Splenocytes were either left untreated (NA) or treated with HU-331 (0.05–2 μM) and/or VH (0.05% ethanol) for 12 h. The intracellular GSH levels were measured by an enzymatic assay described in Section 2. Data are expressed as the mean ± SE for seven samples pooled from three independent experiments. *p < 0.05 as compared with the VH group.

The cytotoxic effect of HU-331 has been shown in several cell lines, including Jurkat T cells, a lymphoma line that is also sensitive to the apoptosis induced by CBD (Kogan et al., 2004; McKallip et al., 2006). Recently, we reported that CBD induced apoptosis in both transformed and non-transformed T cells, including the EL4 thymoma line and primary thymocytes and splenocytes, respectively (Lee et al., 2008; Wu et al., 2008). Although it is currently unknown whether HU-331 induces apoptosis in transformed T cells, it appears that HU-331 and CBD exhibit a similar spectrum of cytotoxic effect in lymphocytes, which includes both transformed and normal T cells.

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Our previous report (Wu et al., 2008) and the present study showed a similar profile of the intracellular changes induced by HU-331 and CBD in splenocytes, including the diminishment of thiols and glutathione (GSH; Fig. 2), and the activation of caspase-8 (Fig. 4). Again, the effective concentration range of HU-331 (0.5–2 μM) is slightly less than that of CBD (4–8 μM). Moreover, the kinetics and magnitude of the effects on thiols and caspase-8 induced by HU-331 and CBD are comparable. Our rationale to focus on thiols is based on the literature evidence showing that HU-331 can form adducts with GSH during hepatic microsomal metabolism of CBD (Bornheim and Grillo, 1998). In addition to GSH, it has been suggested that HU-331 may react covalently with certain amino acid residues containing thiols, such as P450 3A11 (Bornheim and Grillo, 1998). Similar as the induction of thiol diminishment by HU-331, CBD has been demonstrated as a pro-oxidative and pro-apoptotic agent in lymphocytes, in which CBD concurrently induced thiol depletion and ROS generation (Lee et al., 2008; Massi et al., 2006; Wu et al., 2008). Moreover, the thiol diminishment and apoptosis induced by HU-331 in splenocytes can be abrogated by NAC (Tables 1 and 2), a GSH precursor with antioxidative activity. A similar profile of the kinetics between the
thiol diminishment and apoptosis induced by HU-331 suggests an important role for thiols in the apoptosis. The involvement of thiols in HU-331-mediated apoptosis was further confirmed by employing another thiol antioxidant, MPG that also blocked the HU-331-mediated effects. Consistent with these findings on HU-331, NAC completely prevented CBD-mediated apoptosis and thiol diminishment in splenocytes. Collectively, these results suggest that cellular thiols play a pivotal role in the pro-apoptotic effects elicited by both CBD and HU-331.

In addition to thiol antioxidants, two non-thiol antioxidants including catalase and pyruvate were employed to further investigate the pro-oxidative property of HU-331 in splenocytes. Notably, the two non-thiol antioxidants did not influence HU-331-mediated thiol diminishment and apoptosis (Tables 1 and 2). For confirmation of the antioxidative activity of the employed antioxidants, the efficacy of the employed antioxidants to counteract with H2O2-induced thiol diminishment and ROS generation was examined, and the results showed that both thiol and non-thiol antioxidants were effective (Fig. 3). Based on the results that non-thiol antioxidants were effective in counteracting with H2O2-induced ROS generation, but failed to prevent HU-331-mediated thiol diminishment and apoptosis, the involvement of ROS in the HU-331 effects was ruled out. As HU-331-mediated apoptosis can be abrogated by thiol antioxidants, but not non-thiols, it is apparent that HU-331-induced apoptosis is primarily mediated by thiol diminishment. Consistent with this notion, recent reports have shown that salvicine, a diterpenoid quinone compound, triggered apoptosis in HeLa cells via glutathione-depletion-driven mechanisms, including H2O2 generation and topoisomerase II inhibition (Cai et al., 2008; Lu et al., 2005). Similar as HU-331, it was revealed that salvicine diminished the cellular glutathione by direct reaction with glutathione (Cai et al., 2008). Notably, thiols have been proposed as a target to react with HU-331 during the metabolism of CBD with hepatic microsomes (Bornheim and Grillo, 1998). Taken together, these results suggest a differential involvement of thiols and ROS in HU-331-mediated splenocyte apoptosis. It is an intriguing issue to investigate whether the same mechanism applies for CBD.

Although our results suggest that HU-331 might be an active metabolite contributing to CBD-mediated apoptosis in splenocytes, direct evidence showing the metabolism of CBD to HU-331 in splenocytes is lacking. However, as cytochrome P450 3A (CYP 3A) has been reported to be expressed in lymphocytes, macrophages and intestine cells (Dey et al., 2006; Hodges et al., 2000; Starkel et al., 1999; Zhang et al., 2003), we speculate a potential possibility for the metabolism. This issue is currently under investigation at our laboratory.

In summary, the present study demonstrates that HU-331 might be an active metabolite of CBD potentially contributing to the induction of apoptosis in primary lymphocytes. Our mechanistic data suggest a predominant role for cellular thiols, but not ROS, in the induction of apoptosis. As HU-331 is so far one of the most well characterized metabolic derivatives of CBD, our data suggest a potential mechanism for the pro-apoptotic effect of CBD.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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