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A COX-2 metabolite of the endogenous cannabinoid, 2-arachidonyl glycerol, mediates suppression of IL-2 secretion in activated Jurkat T cells

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ARTICLE INFO

Article history:
Received 28 January 2008
Accepted 5 May 2008

Keywords:
2-Arachidonyl glycerol
2-AG
2-AG ether
IL-2
T cell
Cannabinoid
Endocannabinoid
Jurkat
COX
Cyclooxygenase
Anandamide

ABSTRACT

Previous studies from this laboratory have demonstrated that a COX-2 metabolite of the endogenous cannabinoid, 2-arachidonyl glycerol (2-AG), inhibits IL-2 secretion in activated T cells through PPARγ activation independent of the cannabinoid receptors, CB1/CB2. Because numerous cyclooxygenase (COX) products have been shown to activate PPARγ, the primary purpose of the present studies was to determine the role of COX metabolism in the inhibition of IL-2 secretion by 2-AG. Pretreatment with nonselective and COX-2-specific inhibitors completely abrogated 2-AG-mediated suppression of IL-2 secretion. In contrast, pretreatment with COX-1-specific inhibitors had no effect upon 2-AG-mediated inhibition of IL-2 secretion. Interestingly, the current studies also demonstrate that while the potency of 2-AG is comparable between human Jurkat T cells and murine splenocytes, anandamide (AEA) is markedly more potent in suppressing IL-2 production in Jurkat T cells compared to murine splenocytes. Additionally, the present studies also demonstrate that COX-2 protein is readily detectable in resting Jurkat T cells, which is in contrast to resting murine splenocytes in which COX-2 protein is virtually undetectable. Furthermore, COX-2 protein and mRNA levels are significantly increased over basal levels by 2 h following activation of Jurkat cells, whereas increases in COX-2 protein in murine splenocytes are not observed until 4 h after cellular activation. These studies suggest that the potency of AEA in the suppression of IL-2 secretion may correlate with COX-2 protein levels in different T cell models. The present studies are also significant in that they demonstrate 2-AG-mediated inhibition of IL-2 secretion is dependent upon COX-2 metabolism.

1. Introduction

2-Arachidonyl glycerol (2-AG) is an endogenous arachidonic acid derivative and a high-affinity agonist of the cannabinoid receptors, CB1 and CB2, and is hence termed an endocannabinoid [1]. 2-AG has been implicated as an endogenous modulator of the immune system due to its detection in a variety of different cellular elements of the immune system, including dendritic cells, macrophages, microglia, and lymphocytes [2–5]. Moreover, activation of macrophages and other

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0006-2952/$ – see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.bcp.2008.05.005

immune cell types causes a rapid and robust increase in 2-AG levels, suggesting that 2-AG may play a role in immune regulation [4,6–8]. Further evidence for an immunoregulatory role of 2-AG comes from a number of published studies reporting effects by 2-AG in leukocytes and on immunological responses, including calcium influx in HL-60 cells, enhancement of antibody formation in murine splenocytes, and induction of the migration of human peripheral blood monocytes and HL-60 cells [9–11]. In addition, 2-AG also inhibits cytokine release by leukocytes as demonstrated in murine splenocytes, rat microglial cells, and J774 macrophages [12–15].

2-AG has been shown to be hydrolyzed into arachidonic acid and glycerol by two enzymes: monoacylglycerol (MAG) lipase and fatty acid amide hydrolase (FAAH) [16,17]. FAAH is also thought to be the main enzyme responsible for the hydrolysis of anandamide (AEA), another structurally-related ligand of the cannabinoid receptors [18]. In addition to its hydrolysis by MAG lipase and FAAH, 2-AG has also been shown to be metabolized by cyclooxygenase (COX) 2 [19]. COX has two subtypes, 1 and 2, which are similar structurally, but differ in regulation of expression, tissue distribution, and to a certain extent, their substrates [20,21]. While COX-1 is constitutively expressed in most cell types, COX-2 expression is generally restricted to activated leukocytes. Metabolism of arachidonic acid by COX-1 and COX-2 results in the production of a variety of eicosanoids, including prostaglandins and thromboxanes. Like arachidonic acid, 2-AG is also a substrate for COX-2 and PGE synthase and can be metabolized into prostaglandin E2-glyceryl ester, as well as other COX products [22].

Because a number of COX products including cyclopentenone prostaglandins have been found to activate PPARγ and our recent studies have demonstrated that 2-AG treatment results in the suppression of IL-2 secretion through PPARγ activation, the objective of the present studies was to determine the role of COX metabolism in the suppression of IL-2 secretion by 2-AG [23,24]. IL-2 is an autocrine/paracrine factor secreted by activated T cells and is important for T cell survival, proliferation, and in some cases, differentiation. As such, IL-2 is critically involved in the development of an adaptive immune response. We have demonstrated here that impairment of IL-2 secretion by 2-AG is dependent upon COX-2 metabolism. The current studies are the first to demonstrate that inhibition of IL-2 secretion upon 2-AG treatment is mediated by a COX-2 metabolite of 2-AG rather than the parent molecule itself. Moreover, the demonstration that eicosanoids derived from 2-AG modulate lymphocyte function signifies the biological relevance of this new class of COX-2 metabolites.

2. Materials and methods

2.1. Materials

2-Arachidonyl glycerol, SR141716A and SR144528 were provided by the National Institute on Drug Abuse. 2-Arachidonyl glycerol ether, FR122047, SC560, and NS398 were purchased from Cayman Chemical (Ann Arbor, MI). Piroxicam was purchased from Tocris Cookson (Ellisville, MO). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animals and cell culture

Female B6C3F1 mice, 6 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). Studies requiring animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Spleens were isolated aseptically and processed into single-cell suspensions (1×10^6 cells/ml). Cells were cultured in RPMI 1640 supplemented with 100 units penicillin/ml, 100 units streptomycin/ml, 50 μM 2-mercapoethanol (2-ME), and 2% bovine calf serum (BCS). Jurkat E6-1 T cells were purchased from the American Type Culture Collection (Manassas, VA). Jurkat cells were cultured in RPMI 1640 supplemented with 100 units streptomycin/ml, 100 units penicillin/ml, 10 mM nonessential amino acids, 100 mM sodium pyruvate, and 10% BCS.

2.3. IL-2 ELISA

Jurkat cells (5×10^5 cells/ml) or splenocytes (1×10^6 cells/ml) were cultured in triplicate in 48-well culture plates (800 μl/well) in complete RPMI containing 2% BCS. The cells were either pretreated for 30 min with an inhibitor, or left untreated prior to treatment with 2-AG. Following a 30 min incubation, the cells were then stimulated with 40 nM PMA and 0.5 μM ionomycin (PMA/Io). The supernatants were collected 24 h after stimulation and IL-2 protein was quantified using the sandwich ELISA method as previously described [25]. The IL-2 standard (mouse or human recombinant IL-2), purified rat anti-mouse or mouse anti-human IL-2 antibody, and biotinylated anti-mouse or anti-human IL-2 antibody were purchased from BD Pharmingen (San Diego, CA).

2.4. Protein isolation

Jurkat cells (2.5×10^7 cells) or splenocytes (2×10^7 cells) were treated with PMA/Io for 2, 4, 8 or 12 h. At the end of the culture period, the cells were centrifuged and suspended in 100 μl of RIPA buffer (phosphate buffered saline containing 1% Igepal, 0.5% sodium deoxycholate and 0.1% SDS) for 5 min at ambient temperature. The suspension was then sonicated and incubated on ice for 30 min. The cells were then centrifuged at $10{,}000 \times g$ for 20 min at 4 °C and the supernatant was retained. The protein concentration was quantified by BCA assay (Sigma, St. Louis, MO).

2.5. Gel electrophoresis and western analysis

Protein (50 μg Jurkat protein; 30 μg splenocyte protein) was diluted with loading buffer (0.0625 M Tris, 2% SDS 10% glycerol, 0.01% bromophenol blue, and 1% 2-ME), loaded into an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and incubated with blocking buffer (4% dry non-fat milk in 0.05% TBS–Tween 20 for Jurkat protein; also added 1%

bovine serum albumin for splenocyte protein). For the detection of murine COX-2 in the primary splenocytes, a polyclonal anti-mouse COX-2 antibody (Cat # 160106) was purchased from Cayman Chemical (Ann Arbor, MI). Two different primary anti-human COX-2 antibodies were utilized. A monoclonal anti-human COX-2 antibody from Cayman Chemical (Cat # 160112) was compared to an antibody obtained from Dr. David Dewitt (polyclonal rabbit anti-human COX-2). Secondary antibodies were HRP-linked (Amersham Biosciences, Piscataway, NJ). The blots were developed using Supersignal West Femto maximum sensitivity substrate (Pierce Biotechnology, Rockford, IL). Bands were quantified using a densitometer visual imaging system (Bio-Rad, Hercules, CA).

2.6. Real-time PCR

Jurkat cells (5×10^6 cells/ml) were either left untreated or stimulated with PMA/Io for various times. Total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI), following the manufacturer's protocol. The relative expression levels of COX-2 were determined by Taqman real-time PCR using predeveloped primers and probes for COX-2 and 18S ribosomal RNA (Applied Biosystems, Foster City, CA). Relative mRNA expression for COX-2 was calculated as previously described [26].

2.7. Statistical analysis

The mean ± S.E. was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance and the Holm–Sidak or Dunnett's test was used to compare treatment groups to the vehicle (VH) control when significant differences were observed, unless otherwise indicated. Statistical analyses were performed using SigmaStat 3.0 or SuperAnova software.

3. Results

3.1. Suppression of IL-2 secretion by 2-AG, 2-AG ether, and AEA in activated primary splenocytes and Jurkat T cells

Previous studies from this laboratory have shown that treatment with 2-AG or AEA causes a robust suppression of IL-2 secretion in murine splenocytes [12,24,27]. In order to compare the effects of 2-AG and AEA upon human T cells and murine splenocytes, Jurkat T cells and primary splenocytes were treated with 2-AG or AEA spanning a broad range of concentrations. 2-AG treatment of Jurkat T cells produced a marked suppression of IL-2 secretion, which was concentration-dependent, when compared to vehicle (0.1% ethanol), and similar to that observed in murine splenocytes (Fig. 1A). In addition, the effects of 2-AG ether (2-AGE), an ether-linked analogue of 2-AG, were also compared in the two models (Fig. 1B). Interestingly, the inhibition of IL-2 secretion by 2-AGE was significantly greater in human Jurkat T cells when compared to murine splenocytes. Moreover, a more notable difference was observed with AEA treatment, which produced a markedly greater suppression of IL-2 secretion in

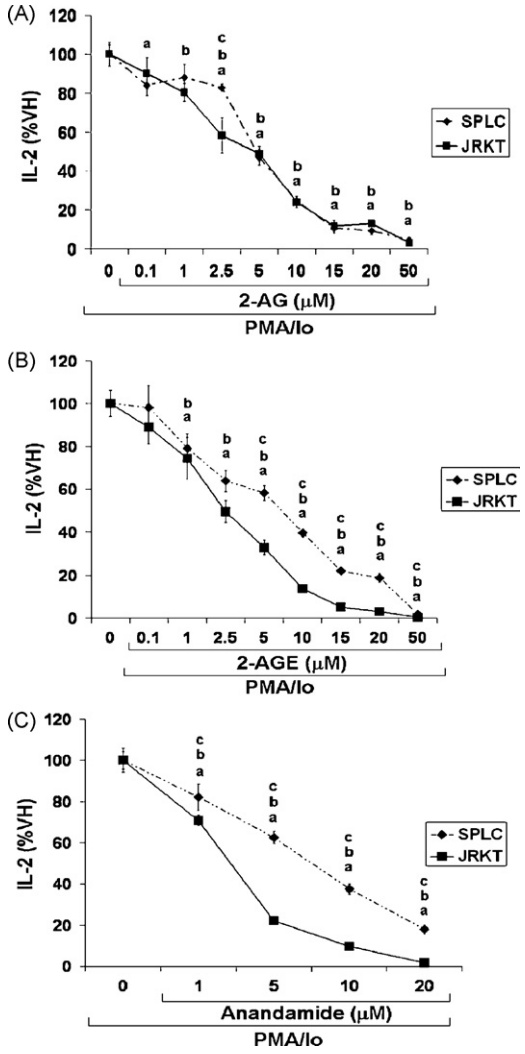

Fig. 1 – Effect of 2-AG, 2-AG ether (2-AGE), and anandamide (AEA) upon PMA/Io-stimulated IL-2 production in activated Jurkat T cells and primary splenocytes. Jurkat T cells (5×10^5 cells/ml) and murine splenocytes (1×10^6 cells/ml) were treated with (A) 2-AG (0.1–50 μM), (B) 2-AGE (0.1–50 μM), (C) AEA (1–20 μM) or vehicle (VH, 0.1% ethanol) for 30 min followed by activation of the cells with 40 nM/0.5 μM PMA/Io. Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA analysis. Cellular viability was ≥85% for all treatment groups as assessed by trypan blue exclusion. The results are presented as % VH and are the mean ± standard error of triplicate cultures. (a) denotes $p < 0.05$ compared to 0 group in SPLC groups, (b) denotes $p < 0.05$ compared to 0 group in Jurkat groups, (c) denotes $p < 0.05$ between the matched SPLC and Jurkat groups. The range of IL-2 induction upon PMA/Io treatment spanned 500–1000 U/ml in splenocytes and approximately 5000–10,000 pg/ml in Jurkat T cells. The background levels of secreted IL-2 were virtually undetectable in both cell preparations.

Jurkat T cells when compared to murine splenocytes (Fig. 1C).

3.2. The effect of the COX inhibitors, flurbiprofen and piroxicam, upon 2-AG-mediated IL-2 suppression

In a prior report from this laboratory, treatment of various cell types with 2-AG was shown to activate PPARγ [24]. Because several COX products have been shown to activate PPARγ, the effects of COX inhibitors upon 2-AG-mediated suppression of IL-2 production were investigated. Pretreatment of Jurkat T cells with flurbiprofen, a nonselective COX inhibitor, completely abrogated suppression of IL-2 secretion by 2-AG (Fig. 2A). In contrast, treatment with piroxicam, an inhibitor that exhibits substantial selectivity for human COX-1 over human COX-2, produced only partial attenuation of 2-AG-mediated IL-2 suppression in Jurkat T cells (Fig. 2B).

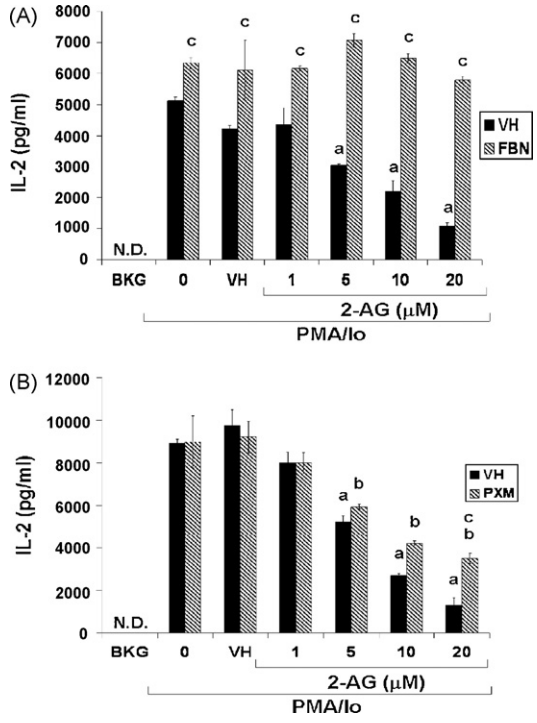

Fig. 2 – Effect of the nonselective COX inhibitor, flurbiprofen, and the COX-1 selective inhibitor, piroxicam, on inhibition of IL-2 secretion by 2-AG. Jurkat T cells (5×10^5 cells/ml) were treated with either (A) 50 μM flurbiprofen (FBN) or (B) 50 μM piroxicam (PXM) for 30 min prior to treatment with 2-AG (1–20 μM) and/or vehicle (VH, 0.1% ethanol) for 30 min. The cells were then stimulated with PMA/Io (40 nM/0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA analysis. Cellular viability was ≥85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. (a) denotes $p < 0.05$ compared to VH + VH group. (b) denotes $p < 0.05$ compared to either (A) FBN + VH group or (B) PXM + VH group. (c) denotes $p < 0.05$ compared to the matched VH group.

3.3. The effect of COX-1- and COX-2-specific inhibitors upon inhibition of IL-2 secretion by 2-AG and 2-AGE

In order to determine which COX isozyme is involved with suppression of IL-2 secretion by 2-AG, specific inhibitors of both COX-1 and COX-2 were employed. Pretreatment with the COX-1-specific inhibitors, SC560 and FR122047, did not attenuate 2-AG-mediated IL-2 suppression (Fig. 3). Although SC560 alone impaired IL-2 secretion, particularly at the highest concentration, it is expected that COX-1 would be inhibited well below this level, since the IC_{50} value for COX-1 inhibition by SC560 is 9 nM. Furthermore, the lack of effect of another potent COX-1-specific inhibitor, FR122047 (IC_{50} = 28 nM), upon 2-AG-mediated suppression of IL-2, strongly suggests that the

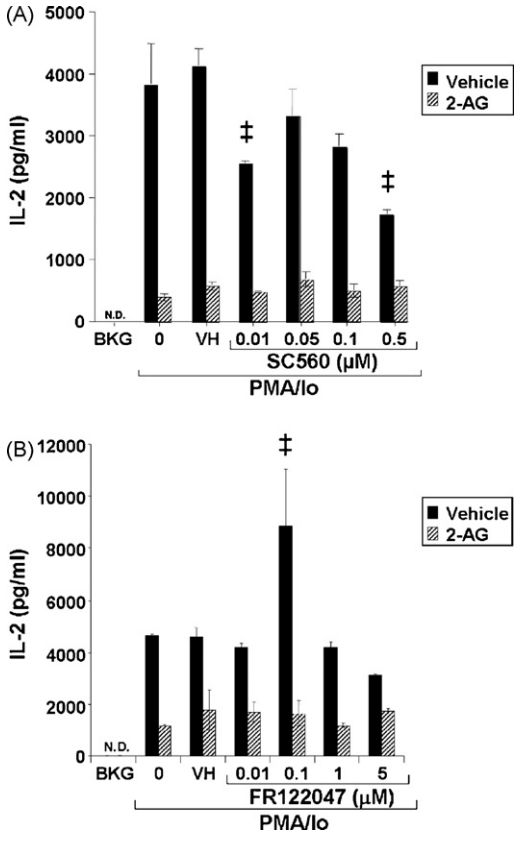

Fig. 3 – Effect of the COX-1-specific inhibitors, SC560 and FR122047, on inhibition of IL-2 secretion by 2-AG. Jurkat T cells (5×10^5 cells/ml) were treated with either (A) SC560 (0.01–0.5 μM) or (B) FR122047 (0.01–5 μM) for 30 min prior to treatment with 2-AG (20 μM) and/or vehicle (VH, 0.1% ethanol) for 30 min. The cells were then stimulated with PMA/Io (40 nM/0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA analysis. Cellular viability was ≥85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. None of the 2-AG + SC560 groups were significantly different from the 2-AG + VH group in panel (A). Likewise, none of the groups treated with both 2-AG and FR122047 were significantly different from the 2-AG + VH control group in panel (B). ‡ denotes $p < 0.05$ compared to the VH + VH group.

suppression of IL-2 secretion by 2-AG occurs independently of metabolism by COX-1. In contrast, pretreatment with a COX-2-specific inhibitor, NS398, caused a concentration-dependent reversal of the suppression of IL-2 secretion by 2-AG (Fig. 4). At the highest concentration of NS398 used (25 μM), 2-AG-mediated IL-2 suppression was completely abrogated, suggesting that the effects of 2-AG upon IL-2 secretion are dependent upon COX-2 metabolism. In contrast, NS398 and FBN had little effect upon suppression of IL-2 production by 2-AGE, suggesting that COX-2 metabolism is not necessary for inhibition of IL-2 by 2-AGE (Fig. 5).

3.4. *COX-2 expression in resting and activated freshly isolated murine splenocytes and human Jurkat T cells*

In order to determine the expression levels of COX-2 protein in freshly isolated murine splenocytes and Jurkat T cells, Western blot analyses were performed. As expected from previously published studies, COX-2 protein levels were virtually undetectable in resting splenocytes but robustly induced following activation of splenocytes with PMA and ionomycin treatment (Fig. 6A) [28]. Increased COX-2 protein was observed as early as 4 h after activation and reached levels as high as 600-fold induction over naïve, untreated cells at 12 h post-activation. In contrast to splenocytes, basal levels of COX-2 protein were readily detected in resting Jurkat T cells, and were further increased as early as 2 h following activation with PMA and ionomycin treatment (Fig. 6B). In addition, modest increases in COX-2 expression were also detected in the absence of PMA and ionomycin treatment over the duration of the culture period. In accordance with the protein levels of COX-2 detected by Western blot analysis in Jurkat cells, increases in COX-2 mRNA were also detected at 2 h post-activation by real-time PCR (Fig. 7).

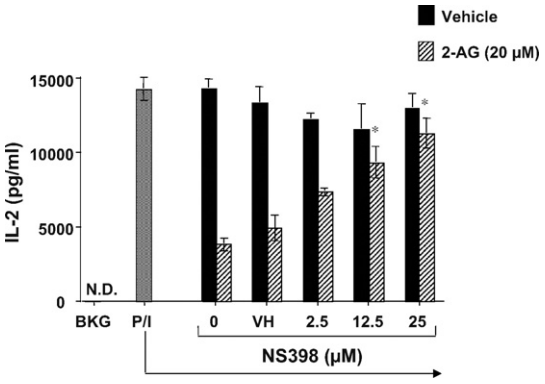

Fig. 4 – Effect of the COX-2-specific inhibitor, NS398, on inhibition of IL-2 secretion by 2-AG. Jurkat T cells (5×10^5 cells/ml) were treated with NS398 (2.5–25 μM) for 30 min prior to treatment with 2-AG (20 μM) for 30 min. The cells were then stimulated with PMA/Io (40 nM/0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA analysis. Cellular viability was ≥85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * denotes $p < 0.05$ compared to the VH + 2-AG group.

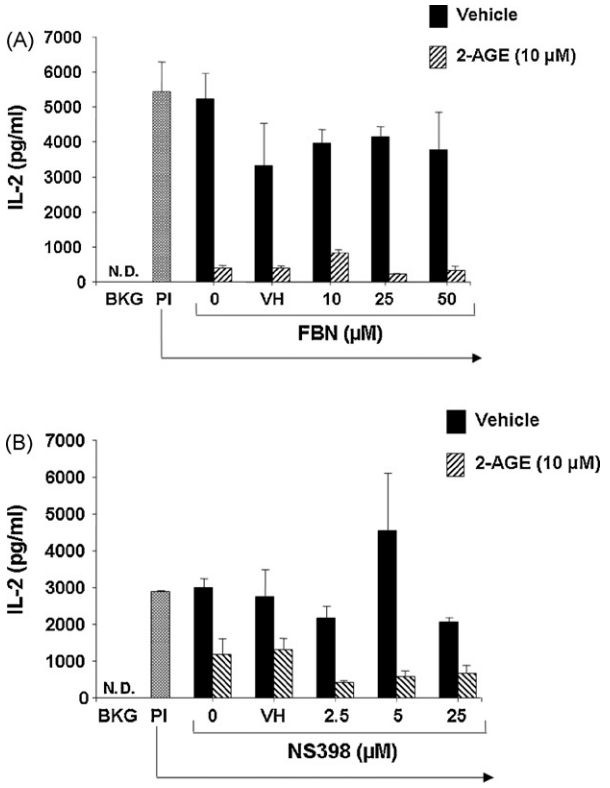

Fig. 5 – Effect of the COX inhibitors, FBN and NS398, on inhibition of IL-2 secretion by 2-AGE. Jurkat T cells (5×10^5 cells/ml) were treated with either (A) FBN (10–50 μM) or (B) NS398 (2.5–25 μM) for 30 min prior to treatment with 2-AGE (10 μM) for 30 min. The cells were then stimulated with PMA/Io (40 nM/0.5 μM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA analysis. Cellular viability was ≥85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. None of the 2-AGE + FBN groups were significantly different from the 2-AGE + VH group in panel (A). Likewise, none of the groups treated with both 2-AGE and NS398 were significantly different from the 2-AGE + VH control group in panel B.

4. Discussion

The present studies are the first to demonstrate that the effects of 2-AG upon IL-2 secretion are dependent upon metabolism by COX-2. These studies are significant in that they suggest that in addition to prostaglandins derived from free arachidonic acid, prostaglandins derived from arachidonic acid derivatives may also modulate immune cell function and thus represent a new class of biologically active eicosanoids. In addition, our previous studies demonstrate that suppression of IL-2 secretion by 2-AG is also dependent upon PPARγ activation, strongly suggesting that the COX-2 metabolite of 2-AG is a PPARγ activator [24]. The current findings are in contrast to a number of published studies showing that inhibition of the COX enzymes potentiates the

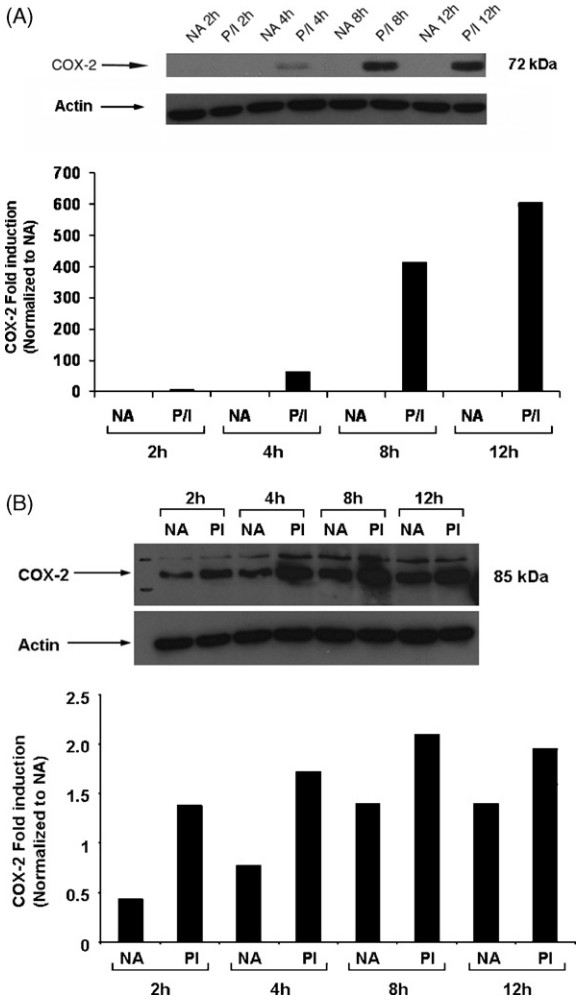

Fig. 6 – Western blot analysis of COX-2 protein levels in resting and activated primary splenocytes and human Jurkat T cells. (A) Freshly isolated murine splenocytes or (B) Jurkat human T cells were either left untreated (NA) or treated with 40 nM PMA and 0.5 μM ionomycin (P/I) for the indicated times (0–12 h). The cells were then lysed and the lysates assayed for COX-2 protein by Western analysis. COX-2 protein levels were quantified by densitometric analysis and normalized to beta-actin protein levels. The results are expressed as fold induction over NA (average value of all NA samples).

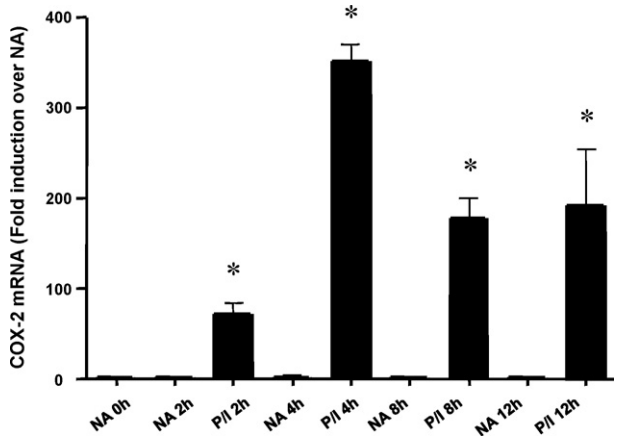

Fig. 7 – Real-time PCR analysis of COX-2 mRNA levels in resting and activated human Jurkat T cells. Jurkat cells were either left untreated (NA) or treated with 40 nM PMA and μM ionomycin (P/I) for the indicated times (0–12 h). The cells were then harvested and the RNA was isolated. COX-2 mRNA levels were detected by real-time PCR analysis and normalized to 18S mRNA levels. The results are expressed as fold induction over the time-matched NA samples. The results are the mean ± standard error of triplicate cultures. * denotes $p < 0.05$ compared to the time-matched NA group as determined by two-tailed Dunnett's analysis.

effects of endocannabinoids in other models [29,30]. While some of the aforementioned studies primarily focused upon CB1-mediated 2-AG effects, previous studies from this laboratory have demonstrated that suppression of IL-2 secretion by 2-AG, 2-AGE, and AEA occurs through activation of PPARγ independently of CB1/CB2 [24,27]. Thus, metabolism of 2-AG by COX enzymes may either increase or decrease the biological activity of 2-AG which is dependent upon whether the cellular target is activated by the parent form of 2-AG (CB1/CB2) or a COX metabolite of 2-AG.

In activated Jurkat T cells, significant suppression of IL-2 by 2-AG is induced at concentrations as low as 1 μM, which is within an order of magnitude of the calculated endogenous 2-AG levels detected in human plasma [31]. Interestingly, the intracellular concentrations of structurally-related congeners, such as AEA, have been reported to exceed extracellular concentrations by as much as three orders of magnitude [32]. While the intracellular concentrations of 2-AG have not yet been reported, they are likely to greatly exceed extracellular levels as 2-AG has been found to accumulate intracellularly by mechanisms similar to or the same as those of AEA [33].

Overall, the increase in COX-2 expression induced by cellular activation was modest in Jurkat cells when compared to primary splenocytes and is likely a result of the higher constitutive levels of COX-2 expression in resting Jurkat cells. Interestingly, while 72–74 kDa is the most frequently reported size for human COX-2, the present studies demonstrate three separate COX-2 immunoreactive bands, ~62, 85, and 170 kDa in Jurkat cells, which have been consistently observed using two different anti-human COX-2 antibodies. The 62 and 85 kDa forms of COX-2 are likely a result of differences in post-translational modifications of the enzyme in Jurkat cells as compared to other human-derived cells. In contrast to the 62 and 85 kDa bands, the 170 kDa band is not inducible by PMA/Io and may represent non-specific binding (Supplemental data). Notably, it has been reported that human COX-2 has at least 4 predicted N-linked glycosylation sites, 13 potential phosphorylation sites, and 9 potential myristoylation sites which may account for differences in molecular weight of human COX-2 isoforms [34,35].

While COX-2 is constitutively expressed and readily detected in resting Jurkat T cells, we show in the present studies that COX-2 is virtually undetectable in resting splenocytes (but upregulated upon activation). Furthermore,

increases in COX-2 protein and mRNA levels are observed as early as 2 h after activation of Jurkat T cells, whereas increases in COX-2 protein expression are not observed until 4 h after activation of murine splenocytes suggesting that the kinetics of COX-2 induction are more rapid in Jurkat T cells than murine splenocytes. The constitutive expression as well as the more rapid kinetics of induction of COX-2 in Jurkat cells may account for the greater sensitivity of Jurkat cells over murine splenocytes to suppression of IL-2 by AEA. While AEA is also a substrate for COX-2, it has been reported that AEA is metabolized by COX-2 much less efficiently than 2-AG or arachidonic acid [22]. Based on these differences in substrate specificity, it is likely that higher levels of COX-2 may be required to fully metabolize AEA and produce the observed suppression of IL-2 secretion than those needed for arachidonic acid and 2-AG. In contrast to AEA, 2-AG is efficiently metabolized by COX-2, such that the low basal levels and slower induction of COX-2 in splenocytes may be sufficient to impair IL-2 secretion to a degree virtually identical to that observed in Jurkat cells.

Although our previous studies have shown that treatment of cells with both 2-AG and 2-AGE activates PPARγ in a number of different models, the present studies demonstrate differences between the two congeners. While NS398 completely abrogates 2-AG-mediated IL-2 suppression, NS398 has little effect upon inhibition of IL-2 by 2-AGE. The aforementioned findings suggest that the effect of 2-AG upon IL-2 secretion requires COX-2 metabolism, while the effects of 2-AGE occur independently of COX-2. Interestingly, it has been reported by Kozak et al. that the prostaglandin glyceryl ester COX metabolites of 2-AG are metabolically more stable than the free acid prostaglandins [36]. Collectively, these observations may suggest that stability is of greater importance than structure in determining the activity of endocannabinoids upon IL-2 secretion. This notion is further supported by the promiscuous nature of the ligand-binding pocket of PPARγ, which is large and thus able to accommodate binding of a number of structurally disparate ligands. An alternate explanation is that both the 2-AG COX metabolite and 2-AGE are metabolized to PPARγ ligands by an enzyme independent, and perhaps downstream, of COX-2.

In addition to differences between 2-AG and 2-AGE in COX metabolism demonstrated by the present studies, other differences in metabolism of the two congeners exist. In contrast to 2-AGE which is non-hydrolyzable, it has been shown that 2-AG is able to be hydrolyzed by FAAH as well as MAG lipase. The inability of MAFP, an inhibitor of FAAH and MAG lipase, to block IL-2 suppression by 2-AG suggests the effects of 2-AG are not dependent upon hydrolysis (unpublished observation). In addition to metabolism by hydrolytic enzymes, numerous studies have demonstrated that 2-AG is efficiently metabolized by COX-2 as well as multiple lipoxygenases [19,37]. While AEA is also a substrate for COX-2, it has been reported that AEA is metabolized by COX-2 much less efficiently than 2-AG or arachidonic acid [22].

A number of oxygenated arachidonate metabolites have been found to activate PPARγ, including the COX product, 15-deoxy-PGJ_2 (15d-PGJ2) [38]. 15d-PGJ_2 is produced through the sequential metabolism of arachidonic acid by COX and PGD synthase, followed by a series of nonenzymatic transformations [39]. It is notable that numerous immune cell types have been shown to synthesize 15d-PGJ_2, including T cells [40]. The activation of PPARγ by 15d-PGJ_2 results in transrepression of transcription factors, such as NFAT, which is ultimately responsible for the suppression of cytokine secretion as well as other immunosuppressive effects [41,42]. In our own studies, we observe a marked and concentration-dependent suppression of IL-2 secretion following direct addition of 15d-PGJ_2 in activated T cells [24]. Despite the variety of immunological effects produced by 15d-PGJ_2, there has been considerable controversy concerning whether it is a biologically relevant PPARγ activator due to the low levels at which it is detected in vivo (low picomolar range) and because 15d-PGJ_2 levels are not elevated under conditions in which PPARγ is activated [43]. While many studies have focused upon the role of free fatty acids and their metabolites as PPARγ activators, our studies suggest that a COX-2 metabolite of 2-AG also activates PPARγ and may be a biologically relevant ligand in cell types known to release 2-AG, such as leukocytes. Although 15d-PGJ_2 is one of the most potent activators of PPARγ, other COX products are also able to activate PPARγ [44]. More studies will be needed to determine exactly which COX products are produced from 2-AG metabolism in activated T cells and which are subsequently responsible for the observed suppression of IL-2 production.

In contrast to arachidonic acid, studies with purified enzyme preparations have shown that metabolism of 2-AG by COX-2 produces a variety of prostaglandin-like compounds that differ from arachidonate-derived products by the addition of a glyceryl ester group. The selective metabolism of 2-AG by COX-2, PGE synthase, PGD synthase, and PGI synthase, coupled with the inefficient metabolism of 2-AG by COX-1, thromboxane synthase, 5-lipoxygenase, as well as other oxidative enzymes, may produce a unique set of eicosanoids tailored to mediate a specific response to 2-AG as opposed to arachidonic acid. Again, part of the mechanism for specific responses to 2-AG might be due to the stability of the prostaglandin glyceryl esters (PG-G) as compared to the free acid prostaglandins [22,36,45].

As compared to AEA, which is also a COX-2 selective substrate, 2-AG is generally found at much higher levels than AEA in most cell types. The release of 2-AG by multiple immune cell types in response to different stimuli suggests that it may play an important role in immune regulation, whereas AEA may be more specialized for particular immune responses. The high levels of AEA found in the uterus, for example, suggest that AEA may function to regulate maternal immune responses in the vicinity of the embryo during gestation [46]. Somewhat related, it has recently been reported that AEA has differential vasorelaxant effects in rat mesenteric vascular beds, depending upon gender, such that tissues derived from female animals are more responsive to the effects of AEA than male tissues [47]. While the present studies have focused upon the effects of endocannabinoids upon primary splenocytes from female mice, it will be interesting in future studies to explore whether such gender-related differences extend to effects on the immune system and inhibition of IL-2 secretion by endocannabinoids.

While the importance of arachidonic acid metabolism by COX-2 in immune cell activation, inflammation, and regula-

tion of immune responses is well established, the present studies suggest that COX-2 also plays a critical role in the metabolism of 2-AG. Furthermore, these studies also suggest that a COX-2 metabolite of 2-AG modulates immune responses of activated T cells and thus may represent an important mechanism of T cell regulation and maintenance of immune homeostasis.

Acknowledgements

This work was supported in part by National Institute of Drug Abuse grants DA12740 and DA15276. We thank Mrs. Kimberly Hambleton for administrative assistance with the preparation of the manuscript. We also thank Mrs. Natasha Snider and Dr. John Buchweitz for technical assistance with the studies. Finally, we thank Dr. David Dewitt for the kind gift of the COX-2 primary antibody.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.05.005.

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