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Title page

15-deoxy $\Delta^{12,14}$ PGJ_2-glycerol ester, a putative metabolite of 2-arachidonyl glycerol, activates peroxisome proliferator activated receptor γ

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Running title page

15-deoxy PGJ_2-G, a putative metabolite of 2-AG, activates PPARγ

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Discussion : 1447

Abbreviations:

2-AG, 2-arachidonyl glycerol; IL-2, interleukin-2; COX-2, cyclooxygenase-2; PPARγ, peroxisome proliferator activated receptor γ; 15d-PGJ_2-G, 15-deoxy-$\Delta^{12,14}$-PGJ_2-glycerol ester; NFAT, nuclear factor of activated T cells; CB1 and CB2, cannabinoid receptor 1 and 2; LPS, lipopolysaccharide; RXR, retinoid X receptor; PPRE, PPAR response elements; NFκB, nuclear factor of the κ-enhancer in B cells; AP-1, activator protein-1; aP2, adipocyte protein 2; AA,

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arachidonic acid; PG, prostaglandin; CGZ, ciglitazone; CsA, cyclosporin A; IFNγ, interferon γ; BSA, bovine serum albumin.

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Abstract

2-Arachidonyl glycerol (2-AG) is an endogenous arachidonic acid derivative capable of suppressing interleukin (IL)-2 production by activated T cells. 2-AG-mediated IL-2 suppression is dependent on cyclooxygenase-2 (COX-2) metabolism and peroxisome proliferator activated receptor γ (PPARγ) activation. The objective of the present studies was to examine if 15-deoxy-$\Delta^{12,14}$-PGJ_2-glycerol ester (15d-PGJ_2-G), a putative metabolite of 2-AG, can mimic the actions of 2-AG on IL-2 regulation through PPARγ activation. 15d-PGJ_2-G bound PPARγ-LBD in a PPARγ competitive binding assay. 15dPGJ_2-G treatment activated PPARγ in a reporter assay, which was attenuated when a PPARγ antagonist, T0070907, was present. 15d-PGJ_2-G treatment suppressed IL-2 production by activated Jurkat cells, which was partially attenuated when pretreated with T0070907. Moreover, IL-2 suppression was pronounced when 15d-PGJ_2-G was present 30 min either before or after T cell activation. Concordant with IL-2 suppression, 15d-PGJ_2-G treatment decreased nuclear factor of activated T cells (NFAT) transcriptional activity in transiently transfected Jurkat cells. Interestingly, T0070907 alone markedly increased NFAT reporter activity suggesting the existence of endogenous PPARγ activation and modulation of NFAT. Because COX-2 metabolism of 2-AG is important for IL-2 suppression, the effect of 2-AG on COX-2 and PPARγ mRNA expression was investigated. 2-AG treatment decreased the upregulation of COX-2 mRNA following T cell activation which suggests negative feedback limiting COX-2 mediated metabolism of 2-AG. PPARγ mRNA expression was increased upon activation and 2-AG treatment produced a modest decrease in PPARγ mRNA expression. Collectively, our findings suggest that 15d-PGJ_2-G activates PPARγ to decrease NFAT transcriptional activity and IL-2 expression in activated T cells.

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Introduction

2-AG is an endogenous arachidonic acid derivative released on demand from membrane precursors (Piomelli, 2003). 2-AG was first isolated from canine gut (Mechoulam et al., 1995; Sugiura et al., 1995) and subsequently has been identified in several tissues and cell types including rat microglial cells (Carrier et al., 2004), neuronal cells (Stella et al., 1997), human platelets, mouse macrophages and human lymphocytes (Berdyshev et al., 2001). Many of the effects of 2-AG have been attributed to cannabinoid receptors, CB1 and CB2, and hence 2-AG was classified as an endocannabinoid (Mechoulam et al., 1995; Sugiura et al., 1995). Within the immune system, 2-AG has been demonstrated to suppress cytokine production, including tumor necrosis factor α release from lipopolysaccharide (LPS)-treated murine macrophages, LPS-treated rat microglial cells, and IL-6 production by J774 macrophages (Berdyshev, 2005). With respect to T cell function, 2-AG produced a concentration-dependent suppression of anti-CD3-induced T cell proliferation and the mixed lymphocyte response (Lee et al., 1995). In addition, 2-AG suppressed IL-2 secretion through impairment of NFAT in activated murine splenocytes (Ouyang et al., 1998) and Jurkat T cells (Rockwell et al., 2006). IL-2 is a critical cytokine for T cell growth and development and is rapidly upregulated upon T cell activation. Although many of the 2-AG-mediated immunomodulatory effects have been attributed to ligation of the CB2 receptor, it has also been previously demonstrated that suppression of IL-2 by 2-AG, anandamide and arachidonate all possessed almost identical IC_{50} values (Rockwell and Kaminski, 2004; Rockwell et al., 2006). Further characterization of 2-AG mediated immune modulation revealed that IL-2 suppression in activated murine splenocytes occurs independently of both CB1 and CB2 receptors and involves, at least in part, the activation of PPARγ (Rockwell et al., 2006).

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PPARγ belongs to the nuclear transcription factor superfamily and usually exists as a heterodimer with retinoid X receptor (RXR). In the resting state, this complex is on the PPAR response elements (PPRE) that are present in the regulatory regions of various target genes and it may or may not exist in association with corepressors depending on the promoter. Ligand binding promotes the dissociation of corepressors (if associated) and the recruitment of coactivators such as SRC1 and CBP/p300. Coactivator recruitment facilitates the integration of histone acetyl transferases (HAT) and thus helps in transcriptional activation of target genes (Glass and Rosenfeld, 2000). In addition to transactivation, ligand-activated PPARγ/RXR also participates in transrepression by physical association with, and sequestration of transcription factors including NFAT, nuclear factor of the κ-enhancer in B cells (NFκB) and activator protein-1 (AP-1), leading to subsequent inhibition of their functions in gene transcription (Ricote et al., 1998; Yang et al., 2000). Although all three PPAR subtypes have been detected in various immune cell types, PPARγ1 expression has been detected in T cells and its activation has been correlated with IL-2 suppression (Clark et al., 2000).

2-AG treatment activates PPARγ as evidenced by increased PPARγ-dependent adipogenesis causing the differentiation of 3T3-L1 fibroblasts into adipocytes, increased mRNA expression of adipocyte protein 2 (aP2), a gene regulated by PPARγ, and increased PPARγ-specific luciferase activity and PPRE binding (Rockwell et al., 2006). It is noteworthy that the 2-AG-mediated IL-2 suppression is dependent on COX-2 metabolism of 2-AG suggesting that a metabolite of 2-AG is responsible for the IL-2 suppression (Rockwell et al., 2008). A comparison of the eicosanoid pathway of arachidonic acid (AA) and 2-AG (which is structurally similar to arachidonic acid) predicts that the potential metabolites of 2-AG are glycerol esters of various prostaglandins and thromboxanes produced from arachidonic acid (Kozak et al., 2002)

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(Fig. 1). One of the putative metabolites of 2-AG is a glycerol ester of a prostaglandin, 15d-PGJ_2-G. Since one of the known agonists for PPARγ is 15-deoxy $\Delta^{12,14}$ PGJ_2 (15d-PGJ_2), the overall objective of the current studies was to investigate whether 15d-PGJ_2-G could mimic the IL-2 suppressive activity of 2-AG through PPARγ activation.

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Materials and Methods

Reagents – 2-AG and anandamide (AEA) was provided by the National Institute on Drug Abuse. Ciglitazone (CGZ), Rosiglitazone (RGZ), 15d-PGJ_2, 15d-PGJ_2-G and T0070907 were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated.

Cell culture – Jurkat cells (clone E6-1, ATCC, Manassas, VA) and Jurkat T-Ag cells (Jurkat T cells stably transfected with large T antigen - a generous gift from Dr. Arthur Weiss, UCSF) were maintained in RPMI 1640 medium supplemented with 10% Bovine Calf Serum (BCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1X solutions of non-essential amino acids and sodium pyruvate (Invitrogen, Carlsbad, CA). HEK 293T (293T) cells were purchased from Open Biosystems and maintained in complete DMEM, consisting of Dulbecco's Modified Eagle Medium supplemented with 10% bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3.7 g/L sodium bicarbonate.

IL-2 ELISA – Jurkat cells were cultured in triplicate (5 x 10^5 cells/ml) in 48-well culture plates (1 ml/well). The supernatants were collected 24 h after phorbol 12-myristate 13-acetate plus calcium ionophore (PMA/I_o - 40 nM/0.5 μM) stimulation, and IL-2 protein was quantified by a sandwich ELISA method as described previously (Kaplan et al., 2003). The IL-2 standard (human recombinant IL-2), mouse anti-human IL-2 antibody and biotinylated anti-human IL-2 antibody were purchased from BD Pharmingen (San Diego, CA).

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Lanthascreen™ TR-FRET PPARγ competitive binding assay – PPARγ binding was assessed according to the manufacturer's instructions (Invitrogen catalog # PV4894). The assay was performed in 384 black microwell plates (Matrical # MP101-1-PP). Briefly, a 20 µl total reaction mixture contained 0.5 nM PPARγ-LBD (GST), 5 nM of TB-anti-GST-tagged antibody, 5 nM of Fluormone™ Pan-PPAR Green, 5 mM DTT (dithiothreitol) and varying concentrations of RGZ, CGZ, 2-AG, AEA and 15d-PGJ_2-G (1 pM - 100µM). The negative control was devoid of the agonist but contained everything else contained in the agonist wells. Following 3-h incubation in the dark, TR-FRET measurements were made in the SPECTRAmax GEMINI XS spectrofluorometer using the following settings: optical module - LanthaScreen™, delay time - 100 µsec, and integration time - 200 µsec. The ratiometric emission (520/490) was plotted against varying agonist concentrations. The data was analyzed using Graph Pad Prism software from GraphPad Software, Inc. (La Jolla, CA) using the sigmoidal curve equation with variable slope to obtain IC_{50} values. The assay quality/robustness score – Z' was calculated and was found to be 0.68 (a value above 0.5 indicates a robust assay).

Plasmids – Human PPARγ-LBD and pFR-luc reporter gene plasmids were a generous gift from Dr. John P. Vanden Heuvel (Pennsylvania State University). The LBD of human PPARγ was fused to the DNA-binding domain of the yeast transcription factor Gal4 under the control of the SV40 promoter. This plasmid was cotransfected with pFR, a luciferase reporter under the control of the Gal4 DNA response element (Vanden Heuvel et al., 2006). NFAT-luc reporter gene plasmid was purchased from Clontech (Mountain View, CA).

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Transient Transfection Assays – For PPARγ reporter assay in HEK293T cells, 2.5 X 10^4 cells per well were preseeded in a 96 well plate in growth medium for 16 to 20 h. The cells were then incubated with the transfection reagents [25 ng of hPPARγ-LBD, 25 ng of pFR-luc and 0.125 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for every well] for 4 h in serum-free medium. After the 4 h incubation, the medium containing the transfection reagents was removed, replaced with complete growth medium, and the cells were allowed to recover for 1 h. Hence, 5 h after transfection, the cells were cultured in the absence or presence of either vehicle (0.02% DMSO) or T0070907 for 30 min, followed by the addition of either 15d-PGJ_2 or 15d-PGJ_2-G (0.1-10 μM). Treatments were performed in triplicate. For Jurkat T-Ag, the cells (5 X 10^5 c/ml) were incubated with transfection reagents (0.75 μg of hPPARγ-LBD, 0.75 μg of pFR-luc and 3 μl of Lipofectamine 2000 for every 5 X 10^5 cells) for 4 h in RPMI 1640 medium with 2% BCS. After the 4 h incubation, the cells were treated with either CGZ (positive control), 15d-PGJ_2 or 15d-PGJ_2-G. Treatments were performed in triplicate. For studies with Jurkat, the cells (5 X 10^5 c/ml) were incubated with transfection reagents (1.5 μg of NFAT-luc and 3 μl of Lipofectamine 2000 for every 5 X 10^5 cells) for 4h in RPMI 1640 medium with 2% BCS. After the 4 h incubation, the cells were cultured in the absence or presence of either vehicle (0.02% DMSO) or T0070907 for 30 min, followed by the addition of cyclosporin A (CsA), CGZ, 15d-PGJ_2 or 15d-PGJ_2-G for 30 min followed by PMA/I_o stimulation (40 nM/0.5 μM). Twenty-four hours after transfection (for HEK293T, Jurkat T-Ag cells and Jurkat cells), luciferase activity was determined using the Luciferase Assay System and Reporter Lysis Buffer (RLB) from Promega (Madison, WI). Protein determinations were performed using a Bicinchoninic Acid Assay (BCA; Sigma, St. Louis, MO).

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Real-Time PCR – Jurkat T cells (5 X 10^6 cells) were treated with 2-AG (20 μM) for 30 min at 37°C, followed by PMA/I_o (40 nM/0.5 μM) stimulation for 2, 4, 8 and 12 h in complete medium containing 2% BCS in 60 mm X 15 mm cell culture dish (Corning Inc, NY). Cells were harvested and RNA isolation was performed using Promega SV Total RNA Isolation System (Promega, Madison, WI). Total RNA was reverse transcribed using random primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). cDNA was amplified with Taqman primers and probe sets (Applied Biosystems) and analyzed using a 7900 HT Fast Real-Time PCR System (Applied Biosystems).

Statistical Analysis – The mean ± SE was determined for each treatment group in the individual experiments. Homogenous data were evaluated by one- or two-way parametric analysis of variance. Dunnett's two-tailed t test was used to compare treatment groups to the VEH control when significant differences were observed using Graph Pad Prism software from GraphPad Software, Inc. (La Jolla, CA) and SigmaStat software (SPSS Inc., Chicago, IL).

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Results

15d-PGJ$_2$-G activates PPARγ-LBD in HEK293T and Jurkat T-Ag cells

A comparison of the eicosanoid pathway of AA and 2-AG suggested that both AA and 2-AG can be metabolized by COX-2 to form PGH$_2$ and PGH$_2$-G (glycerol ester), and depending on the various downstream enzymes, they can form various prostaglandins/thromboxanes and glycerol esters of prostaglandins/thromboxanes, respectively (Kozak et al., 2002) (Fig. 1). One of the putative metabolites of 2-AG is 15d-PGJ$_2$-G, which is the glycerol ester of 15d-PGJ$_2$, a known agonist for PPARγ. 15d-PGJ$_2$-G treatment of HEK293T cells transfected with PPARγ reporter plasmids induced PPARγ-LBD-driven luciferase activity in a concentration-dependent manner (Fig. 2A). 15d-PGJ$_2$ was used as a positive control and also induced PPARγ-LBD-driven luciferase activity in a concentration-dependent manner. It is noteworthy that in the presence of the PPARγ antagonist, T0070907, PPARγ reporter activity was abolished confirming that 15d-PGJ$_2$-G activates PPARγ-LBD (Fig. 2A). Since 15d-PGJ$_2$-G activates PPARγ-LBD in HEK293T cells and it has been demonstrated that 2-AG, other PPARγ agonists, and 15d-PGJ$_2$ suppress IL-2 in activated T cells (Rockwell et al., 2006), we investigated if 15d-PGJ$_2$-G activates PPARγ-LBD in a T cell line. We performed PPARγ reporter assays in Jurkat T-Ag cells because constitutive expression of the large T antigen is essential for the robust expression of the PPARγ-LBD plasmid (Jurkat cells – clone E6-1 lacks large T-antigen). In Jurkat T-Ag cells transfected with PPARγ reporter plasmids, 15d-PGJ$_2$-G induced PPARγ-LBD-driven luciferase activity in a concentration-dependent manner. CGZ and 15d-PGJ$_2$, which are known PPARγ agonists (Forman et al., 1995), also activated PPARγ-LBD in Jurkat T-Ag cells (Fig. 2B).

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15d-PGJ$_2$-G binds to PPARγ-LBD in a PPARγ competitive binding assay

In order to verify direct binding of 15d-PGJ$_2$-G to PPARγ, a Lanthascreen™ TR-FRET PPARγ competitive binding assay was performed. PPARγ agonists, RGZ and CGZ, were used as comparative controls. 15dPGJ$_2$-G bound PPARγ-LBD by competitively displacing the Fluormone™ Pan-PPAR Green and this is evidenced by the decrease in the 520/490 ratio (Fig. 3A). The IC_{50} values were calculated from these concentration response curves and are as follows: RGZ – 74.6 nM, 15d-PGJ$_2$-G – 367.5 nM, and CGZ – 3.45 μM. These results show that 15d-PGJ$_2$-G binds to PPARγ-LBD with lower affinity than RGZ but with higher affinity than CGZ. In addition, the endocannabinoids 2-AG and AEA were also assayed for binding to PPARγ-LBD (Fig. 3B). The determined IC_{50} values were 2-AG – 13.5 μM and AEA – 26.8 μM. The rank order of IC_{50} is RGZ > 15d-PGJ$_2$-G > CGZ > 2-AG > AEA. These results further show that 2-AG itself is a relatively low affinity ligand for PPARγ, especially when compared to 15d-PGJ$_2$-G.

15d-PGJ$_2$-G suppresses IL-2 secretion in a concentration-dependent and time-dependent manner

Since 15d-PGJ$_2$-G bound to and activated PPARγ, the effect of 15d-PGJ$_2$-G-mediated PPARγ activation on IL-2 secretion was investigated in PMA/I_o-stimulated Jurkat T cells. 15d-PGJ$_2$-G treatment produced a robust concentration-dependent suppression of IL-2 secretion compared to the vehicle control (VEH – 0.1% EtOH) (Fig. 4). Treatment with increasing concentrations of 15d-PGJ$_2$ (comparative control) produced a concentration-dependent suppression of IL-2 secretion (Rockwell et al., 2006). CGZ (50 μM used as positive control) also suppressed IL-2 secretion (Clark et al., 2000). Further, we investigated the effect of the presence of 15d-PGJ$_2$-G at various times in relation to stimulation of Jurkat T cells. Time of addition studies in activated

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Jurkat T cells with 15d-PGJ_2-G demonstrated that the presence of 15d-PGJ_2-G either 30 min before or after stimulation of Jurkat cells produced pronounced IL-2 suppression whereas when 15d-PGJ_2-G was added at later time points, 15d-PGJ_2-G-mediated IL-2 suppression was attenuated (Fig. 5). A similar profile was obtained with the presence of CGZ at varying time points. Overall, these results suggest that 15d-PGJ_2-G has to be present early during T cell activation for robust IL-2 suppression.

A PPARγ antagonist, T0070907, attenuates 15d-PGJ_2-G-mediated IL-2 suppression

Since 15d-PGJ_2-G activated PPARγ and caused IL-2 suppression in activated Jurkat cells, we investigated if a PPARγ antagonist, T0070907, attenuated the IL-2 suppression. Pretreatment of Jurkat cells with T0070907 partially attenuated the IL-2 suppression caused by 15d-PGJ_2-G and CGZ. Interestingly, T0070907 (1 μM) in the presence of VEH (0.1% EtOH) produced an increase in IL-2 secretion, suggesting an intrinsic endogenous activity through PPARγ (Fig. 6). It is notable that T0070907 at higher concentrations (2 and 5 μM) did not attenuate 15d-PGJ_2-G-mediated IL-2 suppression and this may be due to other off-target effects of the antagonist (data not shown).

15d-PGJ_2-G decreases transcriptional activity of NFAT and T0070907 alone markedly increases NFAT reporter activity in activated Jurkat cells

One of the most important transcription factors involved in IL-2 gene transcription is NFAT and the increased availability of nuclear NFAT following activation of T cells is responsible for increased IL-2 gene transcription (Clipstone and Crabtree, 1992). Ligation of PPARγ with 15d-PGJ_2 decreased DNA binding of NFAT and caused sequestration of NFAT to PPARγ in human

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peripheral blood T cells (Yang et al., 2000). Since 15d-PGJ_2-G activated PPARγ and decreased PMA/I_o-stimulated IL-2 secretion, we evaluated the effect of 15d-PGJ_2-G on NFAT transcriptional activity. 15d-PGJ_2-G treatment of activated Jurkat cells transiently transfected with NFAT-luc caused a concentration-dependent decrease in PMA/I_o-stimulated luciferase activity (Fig. 7A). CsA, an inhibitor of calcineurin that prevents NFAT dephosphorylation and entry in to the nucleus, was used as a positive control (Clipstone and Crabtree, 1992). Importantly, CGZ also decreases the transcriptional activity of NFAT, suggesting that activation of PPARγ causes a decrease in the transcriptional activity of NFAT (Fig. 7A). Since PPARγ agonists such as troglitazone and 15d-PGJ_2 decreased the transcriptional activity of NFAT (Yang et al., 2000), and we also showed that 15d-PGJ_2-G and CGZ decreased the transcriptional activity of NFAT, we investigated whether a PPARγ antagonist can attenuate the 15d-PGJ_2-G-mediated decrease in NFAT transcriptional activity in activated Jurkat cells. Interestingly, NFAT reporter activity was greatly enhanced with T0070907 (5 and 10 μM) in the presence of the vehicle of agonist (Fig. 7B). The T0070907-mediated enhanced NFAT reporter activity was attenuated in the presence of 15d-PGJ_2-G. It has also been demonstrated earlier that 2-AG decreases NFAT reporter activity in activated Jurkat cells (Rockwell et al., 2006). Importantly, the 2-AG-mediated decrease in NFAT reporter activity was attenuated in the presence of the PPARγ antagonist, T0070907 (Rockwell et al., 2006). These data suggest that the NFAT reporter activity observed after PMA/I_o stimulation is a balance between the reporter activity caused by PMA/I_o stimulation and the suppression of reporter activity caused by the endogenous agonist(s) for PPARγ. In the presence of T0070907, the suppression of the reporter activity caused by PPARγ activation (by the endogenous agonist(s)) may be relieved and hence an increase in NFAT reporter activity was observed.

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2-AG significantly decreases PMA/I_o*-mediated increase in COX-2 mRNA expression but produces only a modest decrease in PPARγ mRNA expression*

COX-2 oxygenates 2-AG as effectively as AA and may lead to the generation of glycerol esters of prostaglandins (Kozak et al., 2002). We have previously demonstrated that PMA/I_o stimulation of Jurkat cells caused an increase in both COX-2 mRNA and protein expression (Rockwell et al., 2008). We now investigated the effect of the presence of 2-AG on COX-2 mRNA expression in activated Jurkat cells. PMA/I_o stimulation increased COX-2 mRNA expression at 2, 4, 8 and 12 h post stimulation as demonstrated earlier (Rockwell et al., 2008). Interestingly, 2-AG treatment (20 μM) significantly decreased PMA/I_o-mediated increase in COX-2 mRNA expression at 2 and 8 h (Fig. 8A). On the other hand, PMA/I_o stimulation of Jurkat cells caused an increase in PPARγ mRNA expression only at 4 h post stimulation and the expression decreased sharply at 8 h and 12 h. 2-AG treatment modestly decreased the PMA/I_o-mediated increase in PPARγ mRNA expression at 4 h, although it was not statistically significant (Fig. 8B). These results suggest the existence of a negative feedback loop to limit the generation of metabolites from 2-AG by COX-2 metabolism and it is tempting to speculate that this feedback inhibition may serve as a checkpoint to limit IL-2 suppression by PPARγ activation.

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Discussion

The major objective of the current studies was to rigorously investigate the role of 15d-PGJ_2-G, a putative metabolite of 2-AG, in mediating IL-2 suppression. The activation of a PPARγ-specific Gal4-responsive reporter by 15d-PGJ_2-G in HEK293T and Jurkat T-Ag cells suggests that this putative metabolite can act as a PPARγ agonist. Importantly, 15d-PGJ_2-G bound PPARγ-LBD with high affinity as evidenced by the IC_{50} value (367.5 nM) compared to that of 2-AG (13.5 μM). This observation implicates that 15d-PGJ_2-G is a higher affinity PPARγ agonist than the parent molecule, 2-AG. In addition, the rank order of IC_{50} for PPARγ agonists is RGZ > 15d-PGJ_2-G > CGZ. These IC_{50} values are concordant with the reported EC_{50} values for RGZ (60 ± 4 nM) and CGZ (3.0 ± 0.7 μM) as evidenced by transactivation assays in CV-1 cells transfected with a chimera consisting of the PPARγ-LBD fused to the Gal4 DNA-binding domain, together with a reporter plasmid containing a GAL4-responsive promoter driving expression of chloramphenicol transferase (Willson et al., 1996). Furthermore, 15d-PGJ_2-G also produced a concentration-dependent decrease in IL-2 secretion and this decrease was pronounced if 15d-PGJ_2-G was present surrounding the time of T cell activation. 15d-PGJ_2-G-mediated IL-2 suppression was partially attenuated in the presence of T0070907, a PPARγ antagonist, suggesting the involvement of PPARγ in the IL-2 suppression. The present studies are the first to demonstrate that a putative metabolite of 2-AG that is downstream of COX-2 metabolism, 15d-PGJ_2-G, might be responsible for the IL-2 suppression observed by 2-AG.

The expression of PPARγ1 has been established in T cells and its activation has been correlated with IL-2 suppression (Clark et al., 2000). Many endogenous ligands of PPARγ have been identified, such as 15d-PGJ_2 (Forman et al., 1995), 5-S-hydroxyeicosatetraenoic acid (15-HETE) (Nagy et al., 1998), polyunsaturated fatty acids (Kliewer et al., 1997), 13-

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oxooctadecadienoic acid (13-OXO) (Bull et al., 2003), 2,4-dienone 13-oxooctadecadienoic acid (13-Oxo-ODE) (Altmann et al., 2007) and components of oxidized low-density lipoprotein, such as 9-hydroxyoctadecadienoic acid (9-HODE) and 13-S-hydroxyoctadecadienoic acid (13-HODE) (Nagy et al., 1998). Of these ligands, 15-HETE is produced by the action of 15-lipooxygenase (15-LOX) on AA whereas 15d-PGJ_2 is produced by the action of COX-2 on AA. We have previously demonstrated that pretreatment with a COX-2 specific inhibitor, NS398, attenuated the 2-AG-mediated IL-2 suppression in activated Jurkat cells (Rockwell et al., 2008). Therefore, we focused our studies on the putative COX-2 metabolite of 2-AG, 15d-PGJ_2-G, which is structurally similar to the known endogenous PPARγ agonist, 15d-PGJ_2.

2-AG is a substrate for COX-2 and there is considerable evidence suggesting the formation of glycerol esters of prostaglandins from 2-AG (Kozak et al., 2002)). The diversity of prostaglandins (PGs) obtained from AA and 2-AG may provide a unique repertoire of mediators customized for specific responses. It has been demonstrated that the glycerol esters of PGs are in general more stable than the free acid prostaglandins, suggesting that the glycerol esters of PGs may have a longer duration of action, albeit it is possible for 15d-PGJ_2-G to undergo hydrolysis to form 15d-PGJ_2 with time. However, these studies are novel in that they suggest that a metabolite rather than the parent molecule itself is responsible for aspects of the immunomodulatory activity mediated by 2-AG.

The ability of 15d-PGJ_2-G to bind, activate PPARγ-LBD and suppress IL-2 secretion in activated Jurkat cells strongly suggests that PPARγ activation is involved in IL-2 suppression. 15d-PGJ_2-G must be present early during T cell activation to robustly suppress IL-2 implicating that PPARγ activation interferes with molecular events active early during IL-2 gene transcription. It has been demonstrated that NFAT can associate with PPARγ when activated

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with PPARγ agonists, troglitazone and 15d-PGJ_2 (Yang et al., 2000). Concordantly, NFAT reporter assays showed decreased reporter activity in activated Jurkat cells when treated with 15d-PGJ_2-G and 2-AG (Rockwell et al., 2006). Importantly, CGZ also decreased NFAT reporter activity suggesting that PPARγ activation leads to a decrease in NFAT transcriptional activity. Together, these results show that PPARγ activation by 15d-PGJ_2-G impairs NFAT function in IL-2 gene transcription. It is noteworthy that in the NFAT reporter system, T0070907 markedly enhanced reporter activity suggesting the existence of endogenous PPARγ activity in activated T cells. Presumably, upon treatment with T0070907, endogenous PPARγ activity is antagonized leading to increased function/release of NFAT that is reflected as increased reporter activity. In agreement with these results, there is a modest increase in IL-2 production in activated Jurkat cells when treated with T0070907 alone at 1 μM. It is consistent with the observed increase in NFAT activity. In another study that is concordant with these results, activated EL4.IL2 T cells treated with the PPARγ antagonist, GW9662, showed increased interferon γ (IFNγ) mRNA levels and protein expression (Cunard et al., 2004). It is notable that NFAT is also critical for IFNγ gene transcription (Macian, 2005). This T0070907-mediated induction of NFAT transcriptional activity is in contrast with our previous studies in which T0070907 enhanced IL-2 production, but had no significant effect on NFAT reporter activity in activated Jurkat cells (Rockwell et al., 2006). The discrepancy might be due to the fact that in the previous studies, T0070907 was present in the culture for only 12 h, whereas in these studies, T0070907 was present for 20 h.

We previously demonstrated that there is a robust increase in COX-2 mRNA and protein expression upon T cell activation (Rockwell et al., 2008). Treatment with 2-AG decreased the increase in COX-2 mRNA caused by T cell activation implicating the existence of a negative

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feedback loop that decreases COX-2 expression after activation, which leads to decreased generation of metabolites, and thus may be limiting IL-2 suppression that is mediated by PPARγ activation. The negative feedback loop may be either PPARγ dependent or independent. There is evidence for inhibition of COX-2 by PPARγ ligands in human cervical cancer cells and it appears to be mediated predominantly through impairment of AP-1 protein binding to the cAMP response element site in the COX-2 promoter (Han et al., 2003). In macrophage-like differentiated U937 cells treated with LPS, 15d-PGJ_2 treatment caused a decrease in COX-2 mRNA expression and COX-2 promoter activity by interfering with the NF-κB signaling pathway, implicating the existence of a negative feedback loop between PPARγ activation and COX-2 expression (Inoue et al., 2000). PPARγ ligands decreased PMA-mediated induction of COX-2 transcription in a concentration-dependent manner in human epithelial cells (Subbaramaiah et al., 2001) and down-regulated COX-2 mRNA and protein expression in human liver cancer cell line HepG2 (Li et al., 2003). In contrast, there is also evidence that a prototypical peroxisome proliferator, WY-14,643, enhanced COX-2 expression in human mammary cells and colonic epithelial cells (Meade et al., 1999). WY-14,643 is primarily an activator of PPARα but it also activates PPARγ (Lehmann et al., 1997) and PPARβ to a lesser extent (Schmidt et al., 1992). Perhaps the increase in COX-2 expression is due to the actions on PPARα and PPARβ. These results taken together suggest that PPARγ generally seems to inhibit COX-2 expression and this effect may be cell-type and/or tissue-type specific. In our study, COX-2 mRNA was decreased by 2-AG treatment beginning at 2 h whereas PPARγ expression was upregulated only at 4 h. Therefore, the decrease in COX-2 mRNA expression may be independent of PPARγ at earlier time points and dependent or independent of PPARγ activation

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during the later time points. More studies are required to understand the molecular mechanisms involved in the negative feedback regulation of COX-2 by PPARγ.

Although the synthetic PPARγ agonist rosiglitazone (Avandia) is currently categorized for restricted use in the management of diabetes, there is mounting evidence that PPARγ activation is involved in immune regulation and in moderating inflammatory responses. PPARγ in T cells has been demonstrated to prevent gut inflammation in mice with experimental inflammatory bowel disease (Guri et al., 2010). Similarly, PPARγ selectively suppressed human and mouse Th17 differentiation (T helper cells secreting IL-17, which play a crucial role in autoimmune diseases such as multiple sclerosis) (Klotz et al., 2009). Likewise, PPARγ has been shown to negatively regulate allergic rhinitis in mice (Fukui et al., 2009). Furthermore, PPARγ also plays an essential role in the control of inflammatory responses by acting on various immune cells such as T cells (Clark et al., 2000), dendritic cells (Angeli et al., 2003), macrophages (Ricote et al., 1998) and mast cells (Sugiyama et al., 2000). Collectively, the aforementioned findings suggest that PPARγ may play an important function in maintaining immune homeostasis and in preventing autoimmune diseases. Further studies are necessary for the identification of the putative metabolite, 15d-PGJ_2-G, within activated T cells and is the focus of ongoing studies in our laboratory. Identification and characterization of the putative metabolite, 15d-PGJ_2-G, will provide important insights into the role of 2-AG and PPARγ in maintaining immune homeostasis.

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Authorship contributions

Participated in research design: Raman, Kaplan, Kaminski, Thompson, Vanden Heuvel

Conducted experiments: Raman

Contributed new reagents or analytic tools: Thompson, Vanden Heuvel

Performed data analysis: Raman

Wrote or contributed to the writing of the manuscript: Raman, Kaplan, Kaminski, Thompson, Vanden Heuvel

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Footnotes

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Legends for Figures

Fig. 1. Comparison of the metabolic pathway of arachidonic acid (AA) and 2-arachidonyl glycerol (2-AG). AA and 2-AG can be metabolized by COX-2 to form PGH_2 and PGH_2-G, respectively. Depending on the downstream enzymes, AA and 2-AG can be further metabolized to form various prostaglandins and their glycerol esters, respectively. 15-deoxy-$\Delta^{12,14}$ PGJ_2 is a known endogenous agonist for PPARγ and it is hypothesized that 15-deoxy -$\Delta^{12,14}$ PGJ_2-G (the glycerol ester of 15-deoxy-$\Delta^{12,14}$ PGJ_2) may act as an agonist for PPARγ. PG, Prostaglandin; TX, Thromboxane; COX-2, cyclooxygenase-2; PPARγ, peroxisome proliferator activated receptor γ.

Fig. 2. PPARγ-LBD reporter activity in HEK293T and Jurkat T-Ag cells treated with 15d-PGJ_2-G. (A) HEK293T cells (2.5 X 10^4 cells per well) were preseeded in a 96 well plate in growth medium for 16 to 20 h. The cells were then transiently transfected with hPPARγ-LBD and pFR-luc. After transfection, the cells were cultured in the absence or presence of either vehicle (0.02% DMSO) or T0070907 for 30 min, followed by the addition of vehicle (VEH of agonist - 0.1% EtOH), 15d-PGJ_2 or 15d-PGJ_2-G (0.1-5 μM). 24 h after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist alone and †, $p < 0.05$ compared to VEH for T0070907 within each treatment group. The results are representative of three separate experiments. (B) Jurkat T-Ag cells (5 X 10^5 c/ml) were transiently transfected with hPPARγ-LBD and pFR-luc using Lipofectamine 2000 for 4 h in RPMI 1640 medium with 2% BCS in a 48 well plate. After the 4 h incubation, the cells were either left untreated (NT – No treatment) or treated with CGZ, 15d-PGJ_2, 15d-PGJ_2-G

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or vehicle (VEH of agonist - 0.1% EtOH). 24 h after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist. The results are representative of three separate experiments.

Fig. 3. Lanthascreen™ TR-FRET PPARγ competitive binding assay. (A) Effect of 15d-PGJ2-G, CGZ and RGZ. (B) Effect of 2-AG and AEA. The reaction mixture contained 0.5 nM PPARγ-LBD (GST), 5 nM of TB-anti-GST-tagged antibody, 5 nM of Fluormone™ Pan-PPAR Green, 5 mM DTT (dithiothreitol) and varying concentrations of RGZ, CGZ, 15d-PGJ_2-G, 2-AG and AEA (1 pM - 100μM). Following 3-h incubation in the dark, TR-FRET measurements were made in the SPECTRAmax GEMINI XS spectrofluorometer. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the TR-FRET ratio of respective vehicle control (VEH of RGZ – 5% EtOH – 0.43063, VEH of CGZ and 15dPGJ_2-G – 1% EtOH – 0.44179, VEH of 2-AG and AEA – 1% EtOH – 0.43342). The results are representative of three separate experiments.

Fig. 4. Effect of 15d-PGJ_2-G upon IL-2 secretion by activated Jurkat cells. Jurkat cells (5×10^5 cells/ml) were either left untreated (NT – No treatment) or treated with CGZ (50 μM), 15d-PGJ_2 (0.1 – 10 μM), 15d-PGJ_2-G (0.1 – 10 μM) or vehicle (VEH of agonist – 0.1% EtOH) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μM ionomycin (P/I) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist. The results are representative of three separate experiments.

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Fig. 5. Time of addition studies with 15d-PGJ_2-G in activated Jurkat cells. At time -30 min to 8 h post stimulation with PMA/I_o (P/I), Jurkat cells (5 x 10^5 cells/ml) were treated with CGZ (50 μM) or 15d-PGJ_2-G (5 μM) or vehicle (VEH of agonist – 0.1% EtOH). 24 h after stimulation, the supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the time-matched VEH control and †, $p < 0.05$ compared to the respective – 30 and + 30 min treatment. The results are representative of three separate experiments.

Fig. 6. Effect of T0070907 on 15d-PGJ_2-G-mediated IL-2 secretion by activated Jurkat cells. Jurkat cells (5 x 10^5 cells/ml) were treated with vehicle of T0070907 (VEH of antagonist - 0.02% DMSO) or (0.1 and 1 μM) for 45 min, followed by the addition of CGZ (10 or 20 μM), 15d-PGJ_2-G (2.5 or 5 μM) or vehicle (VEH of agonist – 0.1% EtOH) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μM ionomycin (P/I) for 24 h. The supernatants were harvested and IL-2 production was measured by ELISA. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to (VEH of agonist + VEH of antagonist) and †, $p < 0.05$ compared to VEH of T0070907 within each treatment group. The results are representative of three separate experiments.

Fig. 7. NFAT reporter activity in Jurkat cells treated with 15d-PGJ_2-G in the (A) absence and (B) presence of a PPARγ antagonist, T0070907. Jurkat cells (5 X 10^5 c/ml) were transiently transfected with NFAT luciferase reporter using Lipofectamine 2000 for 4 h in RPMI 1640 medium with 2% BCS in a 48 well plate. In (A) after the 4 h incubation, the cells were either left untreated (NT - No treatment) or treated with vehicle (VEH of agonist - 0.1% EtOH), CsA

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(0.01 μM), CGZ (50 μM), or 15d-PGJ_2-G (5 and 10 μM) for 30 min followed by PMA/I_o stimulation (P/I - 40 nM/0.5 μM). In (B) after the 4 h incubation, the cells were treated with vehicle of the antagonist (0.02% DMSO) or T0070907 (5 and 10μM) for 30 min. Then the cells were treated with vehicle (VEH of agonist - 0.1% EtOH), or 15d-PGJ_2-G (2.5 – 5 μM) for 30 min followed by PMA/I_o stimulation (P/I - 40 nM/0.5 μM). 24 h after transfection, the luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. The results are the mean ± S.E. of triplicate cultures. In (A) statistical significance is indicated by *, $p < 0.05$ compared to VEH of agonist. In (B) statistical significance is indicated by *, $p < 0.05$ compared to (VEH of agonist + VEH of T0070907) and †, $p < 0.05$ compared to VEH for T0070907 within each treatment group. The results are representative of three separate experiments.

Fig. 8. Real-time PCR analysis of (A) COX-2 mRNA and (B) PPARγ mRNA levels in resting and activated Jurkat T cells treated with 2-AG. Jurkat cells were left untreated (NT - No treatment), treated with vehicles (VEH of 2-AG - 0.1 % EtOH; VEH of PMA/I_o- 0.1 % DMSO) or 2-AG (20 μM) in the presence or absence of 40 nM PMA and 0.5 mM ionomycin (P/I) for the indicated times (0 – 12h) after which the RNA was isolated. COX-2 mRNA and PPARγ mRNA levels were detected by real-time PCR analysis and normalized to 18S mRNA levels. The results are expressed as fold induction over the NA samples at 0 h. The results are the mean ± S.E. of triplicate cultures. Statistical significance is indicated by *, $p < 0.05$ compared to the time-matched EtOH + P/I. The results are representative of three separate experiments.

Fig. 1

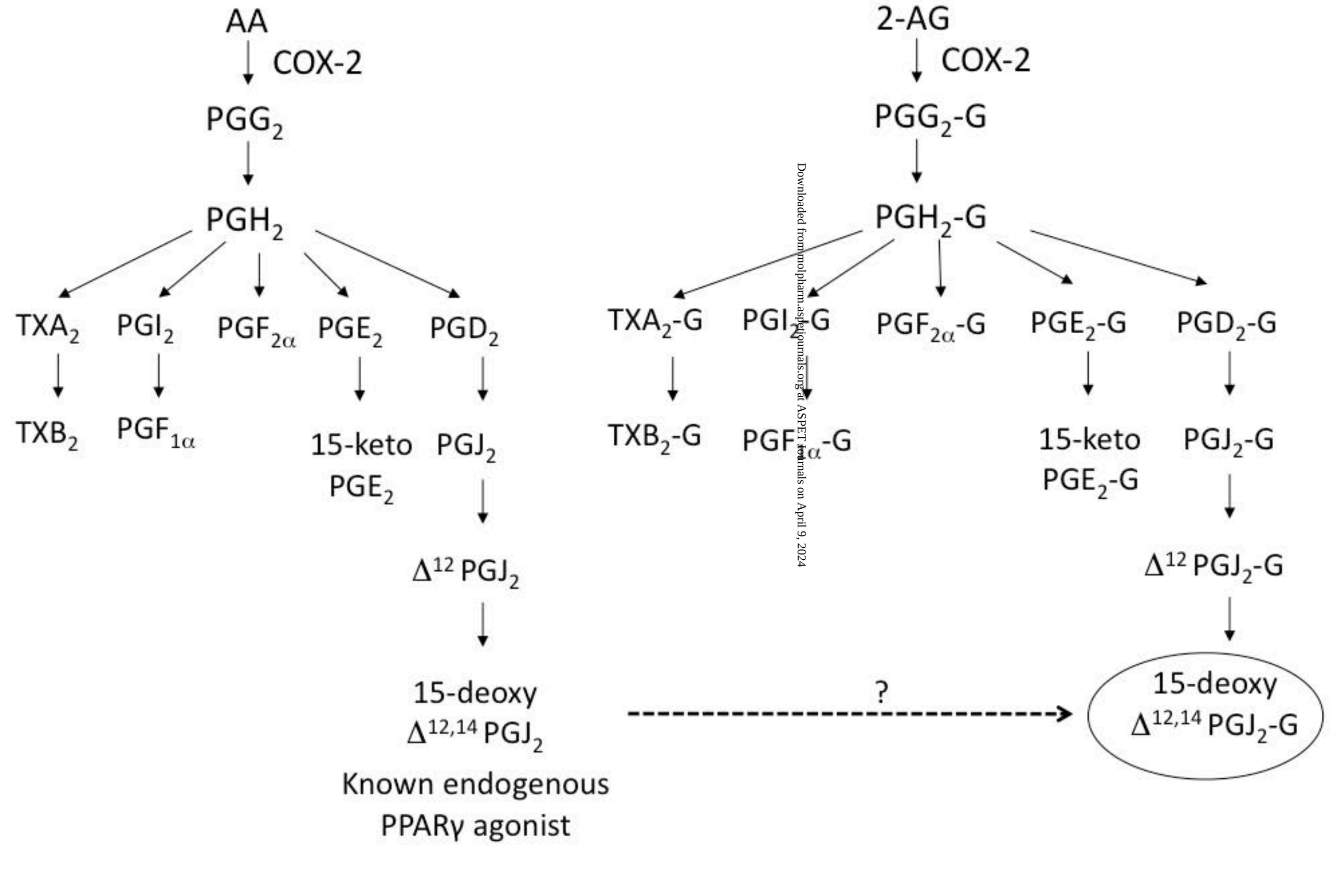
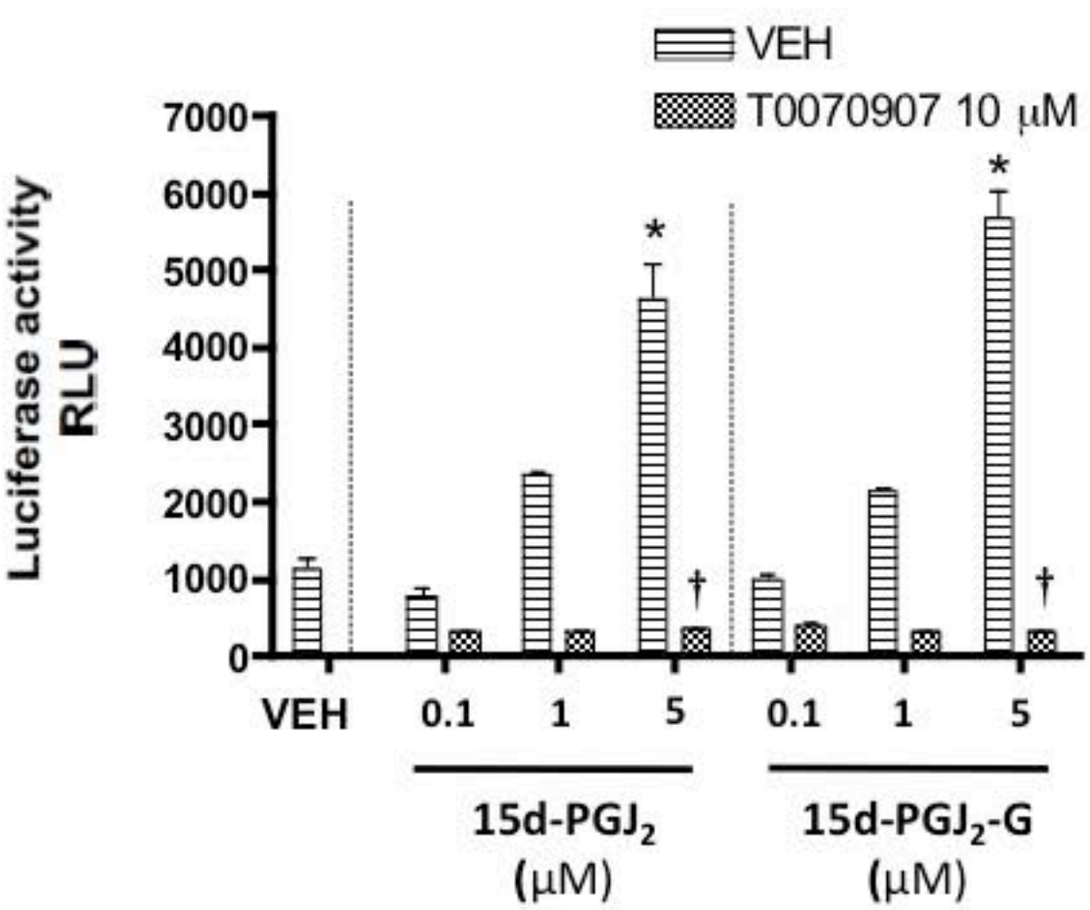


Fig. 2 (A)



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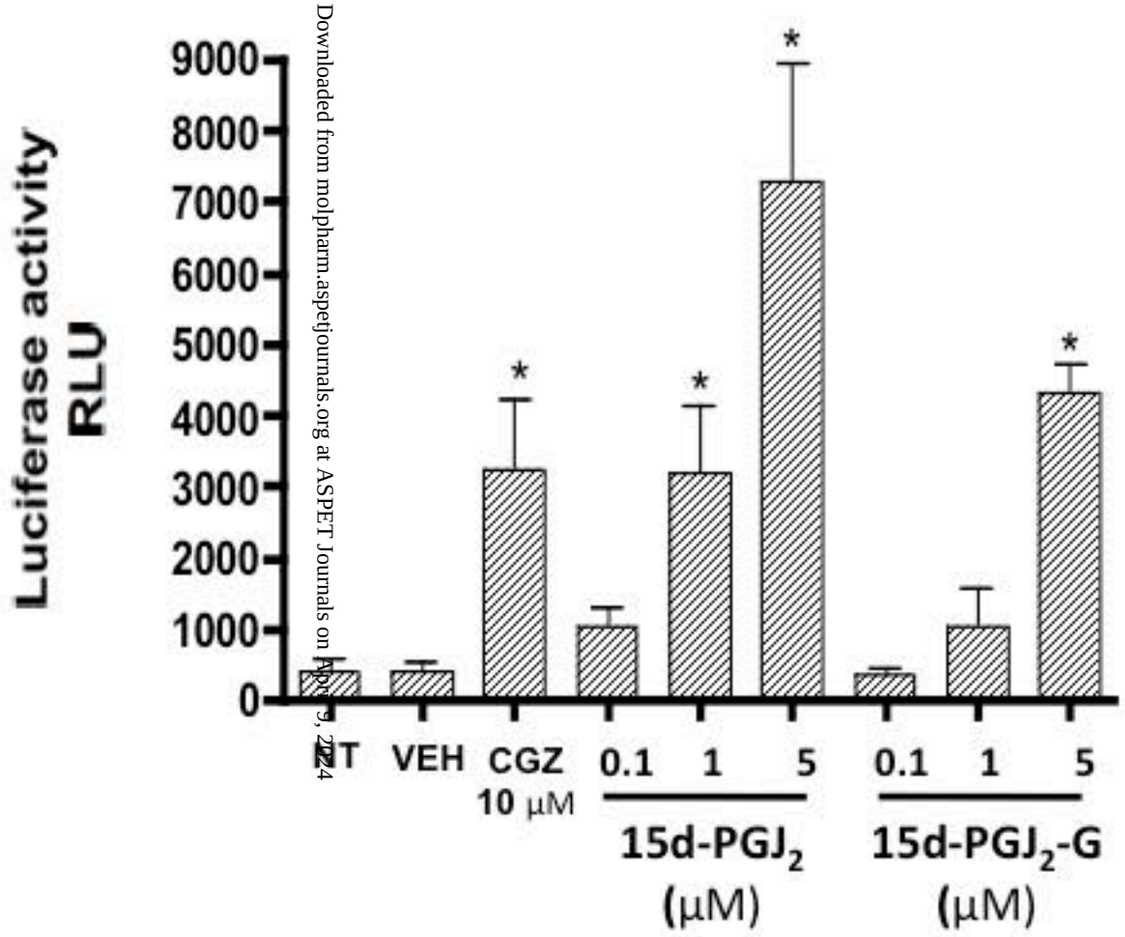

Fig. 3 (A)

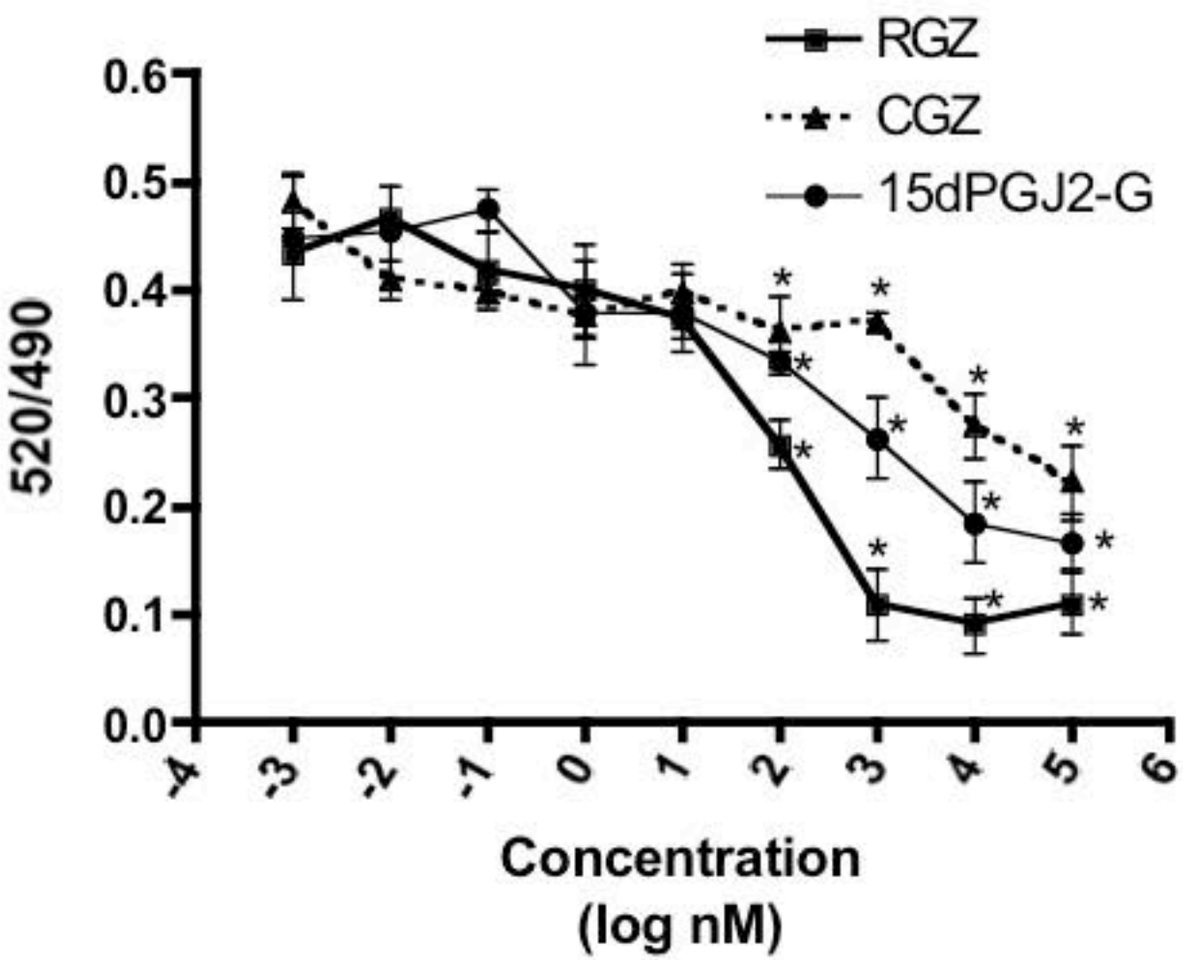

Fig. 3 (B)

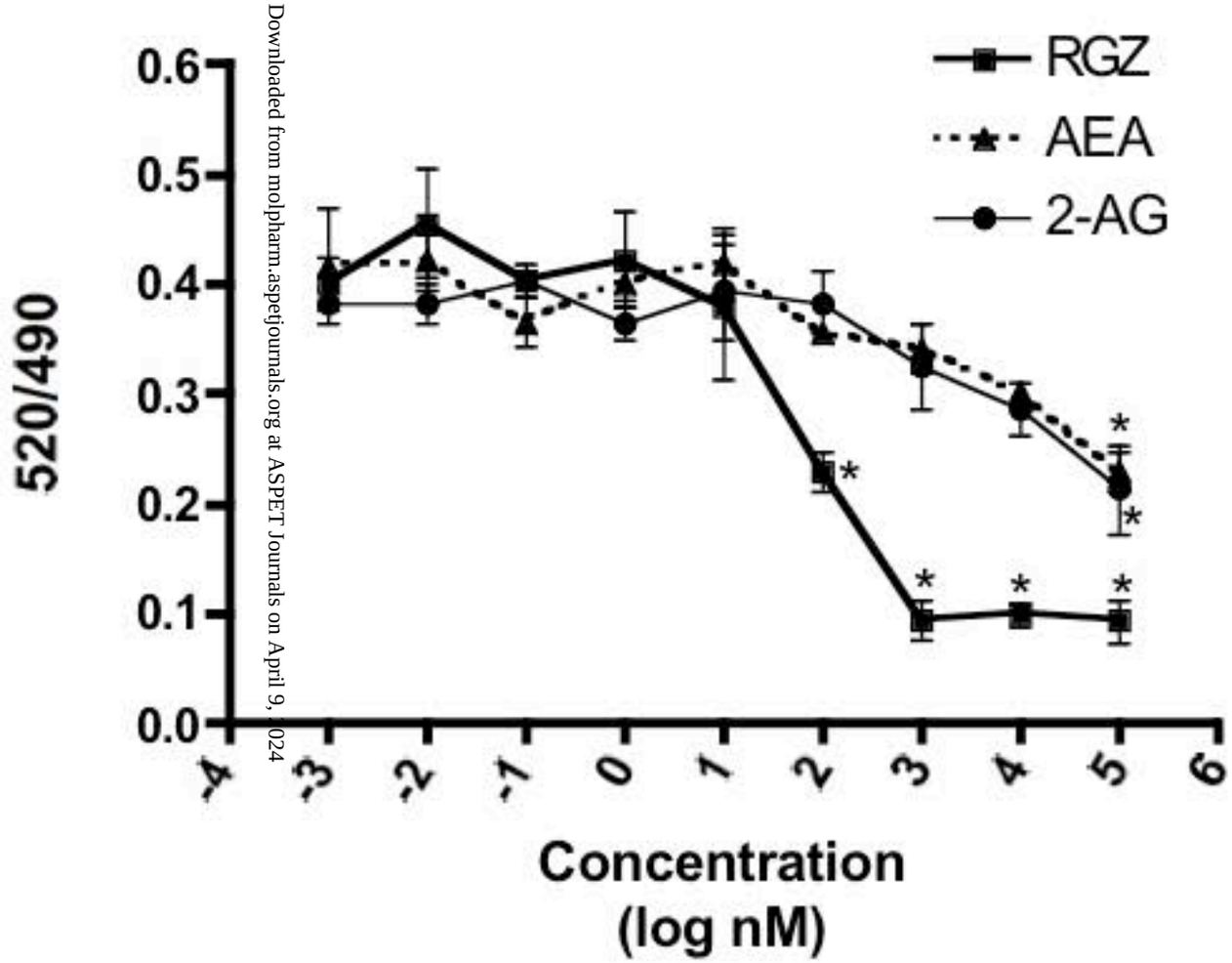

Fig. 4

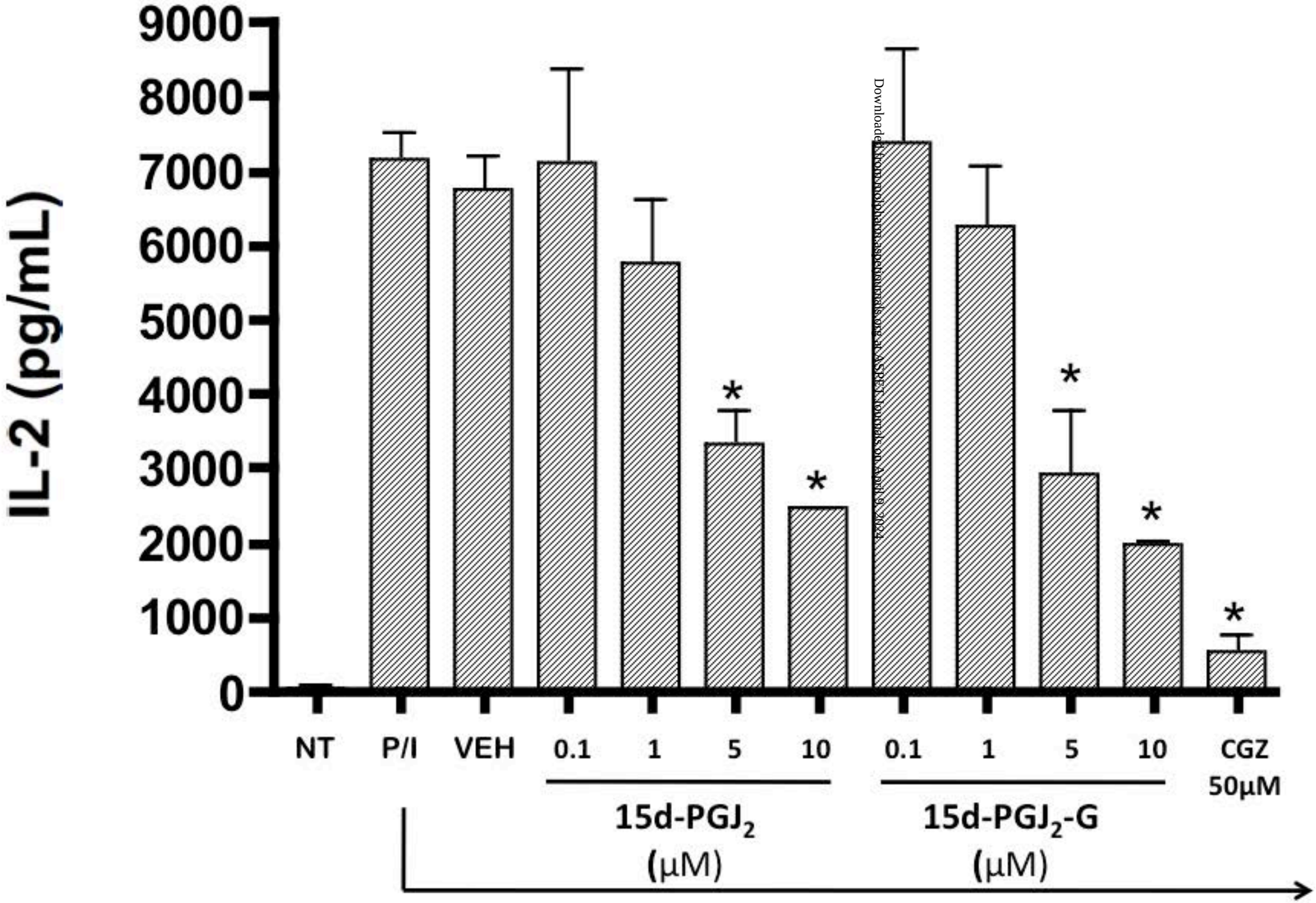

Fig. 5

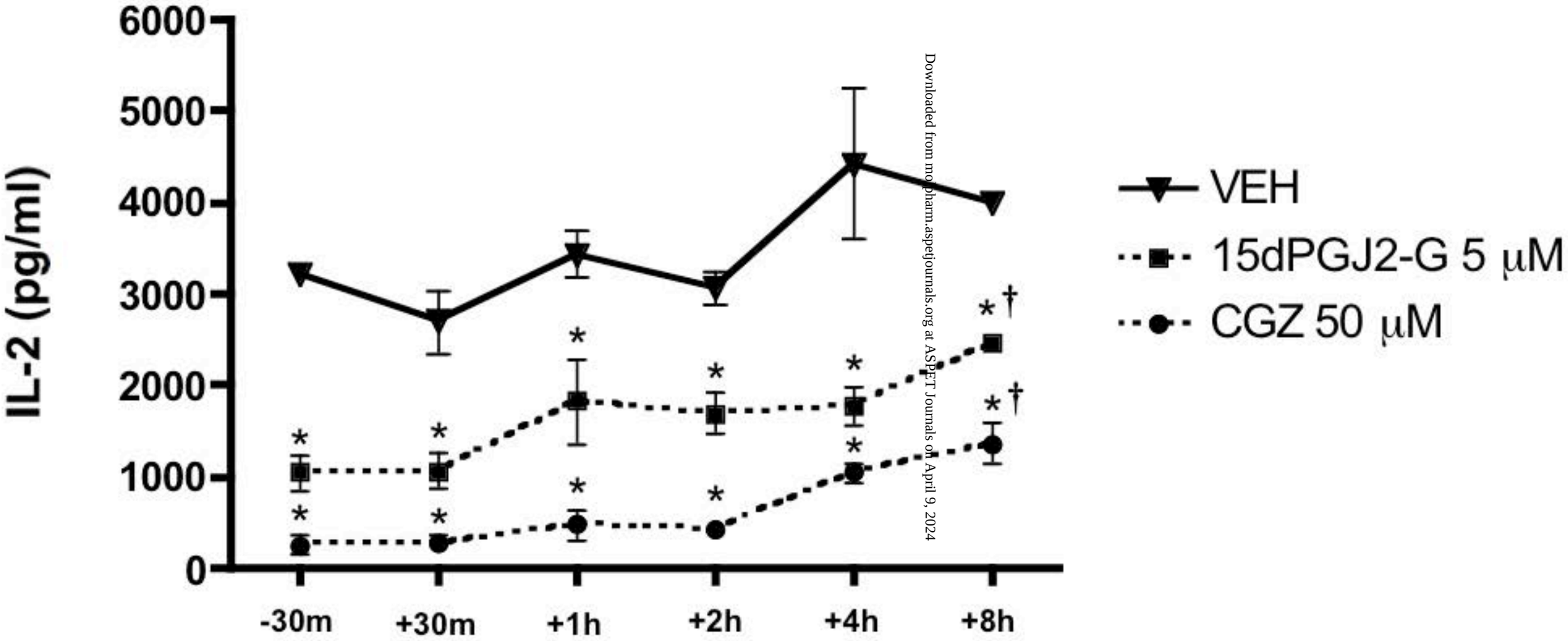

Fig. 6

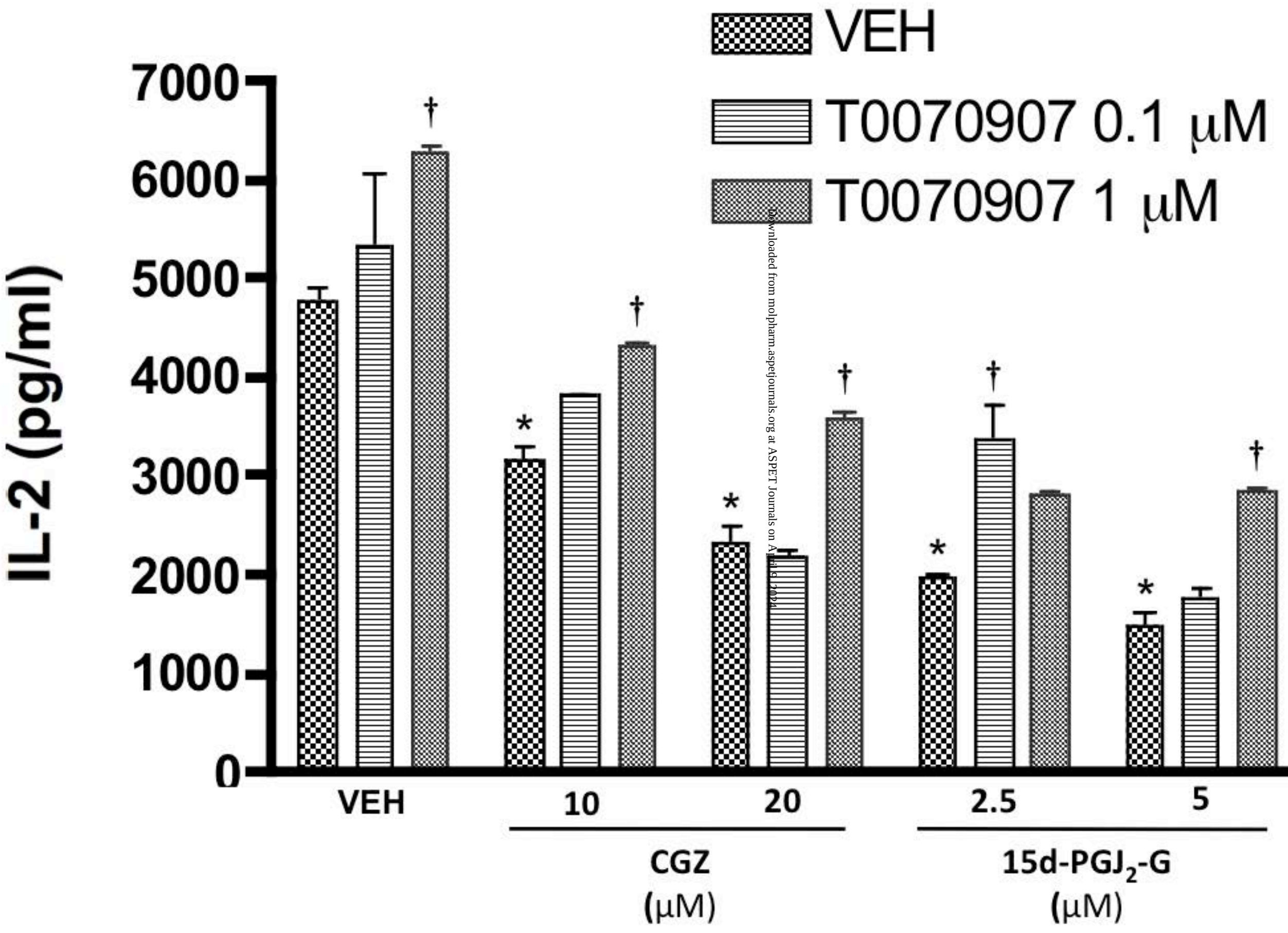

Fig. 7 (A)

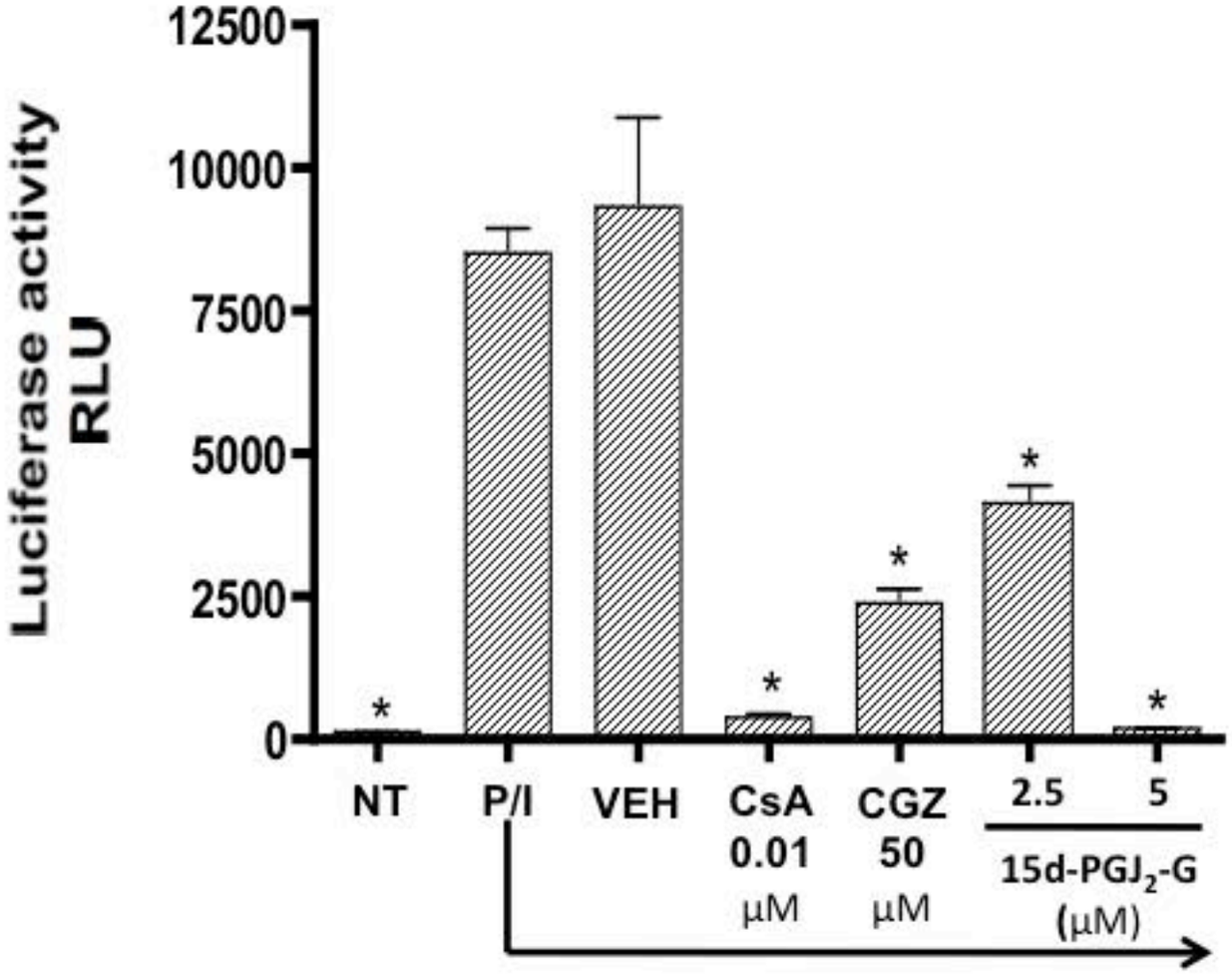

Fig. 7 (B)

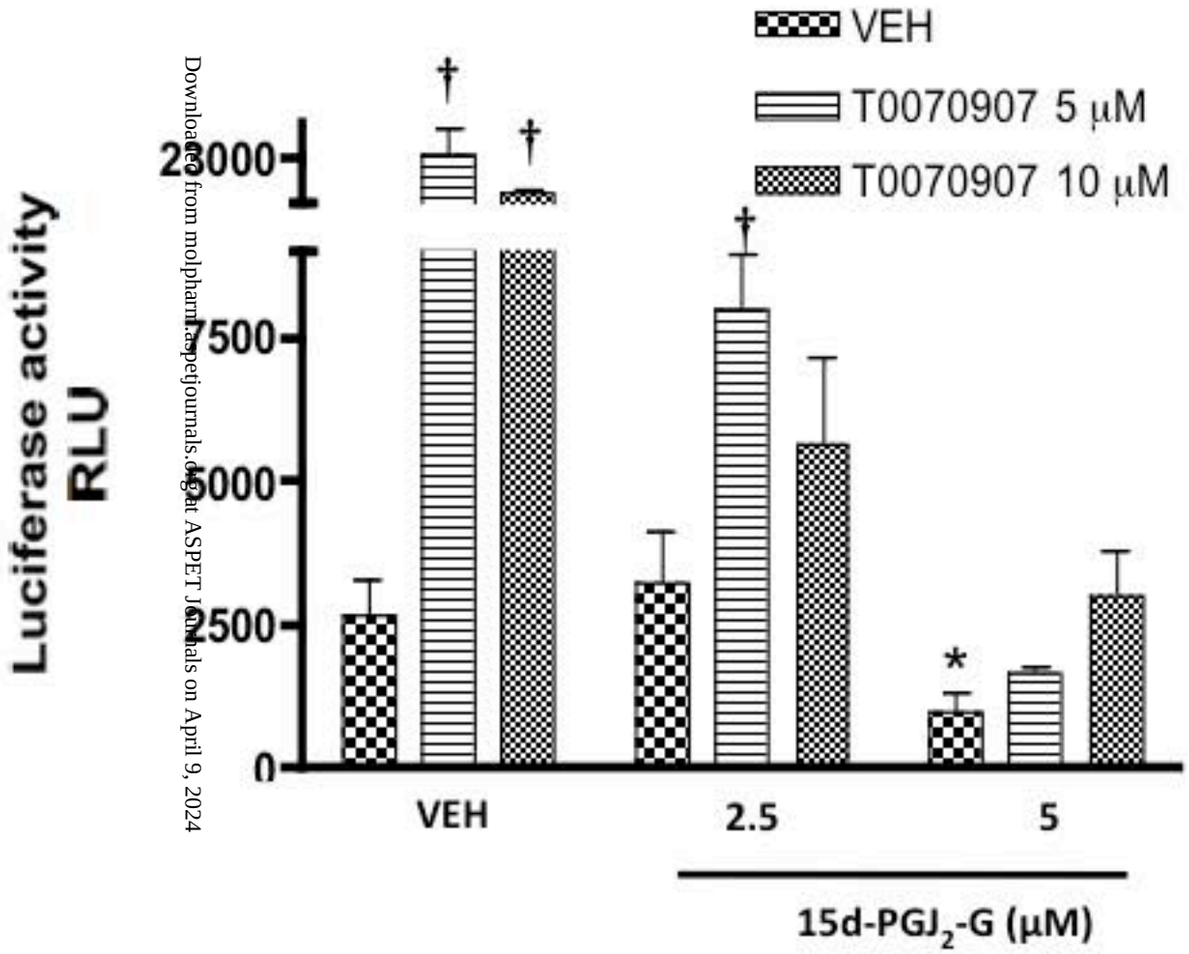

Fig. 8 (A)

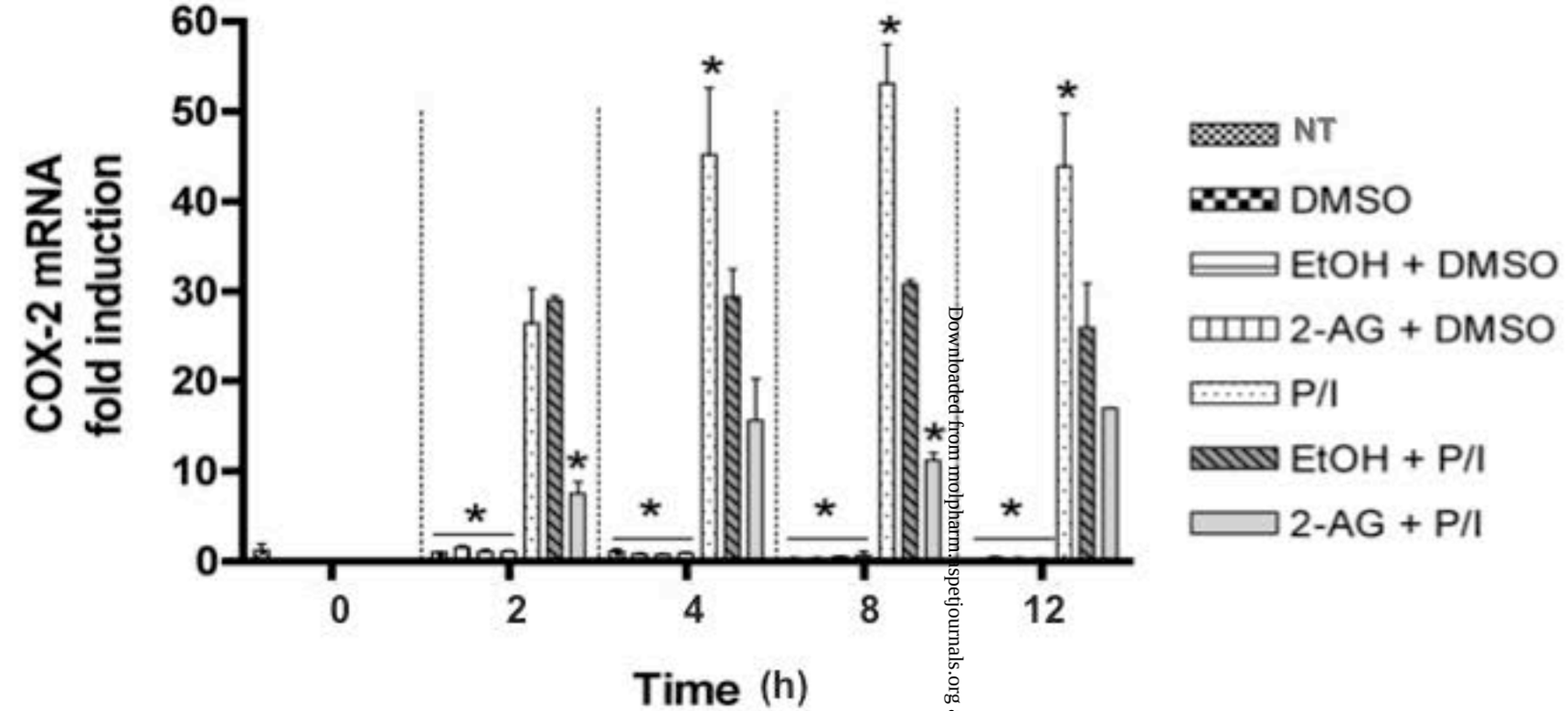

Fig. 8 (B)