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A CYCLOOXYGENASE METABOLITE OF ANANDAMIDE CAUSES INHIBITION OF INTERLEUKIN-2 SECRETION IN MURINE SPLENOCYTES

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Running title: Anandamide inhibits interleukin-2 secretion

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Abbreviations: AMT, anandamide membrane transporter; CB1, cannabinoid receptor 1, CB2, cannabinoid receptor 2, FAAH, fatty acid amidohydrolase; FR122047, 1-[[4,5-bis(4-Methoxyphenyl)-2-thiazolyl]carbonyl]-4-methylpiperazine; MAFP, methyl arachidonyl fluorophosphonate; NS398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; PMA, phorbol 12-myristate 13-acetate; SC560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)3-3(trifluoromethyl)1H-pyrazole; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorphenyl)-4-methyl-H-pyrazole-3 carboxyamidehydrochloride; SR144528, N-[(1S)-endo-1,3,3,-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; T0070907, 2-chloro-5-nitro-N-4-pyridinyl-benzamide; UCM707, N-(3-furanylmethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; VR1, vanilloid receptor 1

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Abstract

Arachidonyl ethanolamine, which is commonly known as anandamide, was the first endogenous compound to be identified which binds to the cannabinoid receptors. Anandamide mimics many of the physiological effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), including hypothermia, antinociception, immobility, catalepsy, and immune modulation. In the present studies we show that anandamide caused a concentration-dependent inhibition of interleukin-2 in primary splenocytes. The CB1 and CB2 antagonists, SR141716A and SR144528, when used in combination, did not antagonize the inhibition of interleukin-2 by anandamide. Additionally, neither UCM707, the inhibitor of the putative anandamide membrane transporter (AMT), nor methyl arachidonoyl fluorophosphonate (MAFP), the inhibitor of fatty acid amidohydrolase (FAAH), were able to affect the inhibitory activity of anandamide upon interleukin-2. Interestingly, arachidonic acid caused a concentration-dependent inhibition of interleukin-2 secretion (IC_{50}=10.3 μM), which was similar to that of structurally-related anandamide (IC_{50}=11.4 μM). The inhibition of interleukin-2 by anandamide and arachidonic acid was partially reversed by pretreatment with the nonspecific cyclooxygenase inhibitors, flurbiprofen and piroxicam. Moreover, NS398, a cyclooxygenase-2 specific inhibitor, also attenuated the inhibitory effects of anandamide and arachidonic acid upon interleukin-2 secretion. Finally, pretreatment with a PPARγ-specific antagonist, T0070907, partially antagonized anandamide-mediated suppression of IL-2 secretion. Collectively, the aforementioned studies suggest that inhibition of interleukin-2 secretion by anandamide is independent of CB1/CB2 and the AMT/FAAH system. Additionally, these studies also suggest that inhibition of interleukin-2 is

mediated by a PPARγ, which is activated by a cyclooxygenase-2 metabolite of anandamide.

Introduction

Two cannabinoid receptors have been isolated and cloned to date, CB1 and CB2 (Matsuda et al., 1990; Munro et al., 1993). CB1 is widely distributed in the central nervous system, but is also found in a number of other tissues (Matsuda et al., 1990). CB2 is the predominant cannabinoid receptor expressed in cells of the immune system, although CB1 is also detected in many immune cells at lower levels (Kaminski et al., 1992; Munro et al., 1993). Both receptors have seven transmembrane domains and are G-protein coupled. CB1 and CB2 share only 44% homology, which increases to 68% when comparing only the transmembrane regions that contain the ligand-binding domains (Munro et al., 1993). Arachidonyl ethanolamine, also known as anandamide, was the first endogenous cannabinoid receptor ligand to be identified through radioligand binding analysis and was therefore termed an endocannabinoid (Devane et al., 1992).

Recently published studies suggest that CB2 may be involved with a number of immunomodulatory effects, including inhibition of macrophage-dependent T cell activation, induction of the immunosuppressive cytokine, transforming growth factor β, in human peripheral blood lymphocytes stimulated with anti-CD3, inhibition of antigen processing by macrophages, and induction of cytokine and chemokine production in HL-60 cells (McCoy et al., 1999; Buckley et al., 2000; Derocq et al., 2000; Gardner et al., 2002). Although CB1 is typically expressed at much lower levels in immune cells than CB2, there is also evidence to suggest a role for CB1 in certain immunological effects, such as inhibition of tumor necrosis factor α and interleukin-12 in LPS-treated mice as well as induction of interleukin-6 in mouse astrocytes (Molina-Holgado et al.,

1998; Smith et al., 2001). Anandamide has been shown to modulate a number of immunological responses, including inhibition of nitric oxide and cytokine production in macrophages as well as inhibition of tumor necrosis factor α and neutrophil recruitment in lipopolysaccharide-induced pulmonary inflammation in mice (Berdyshev et al., 1998; Chang et al., 2001). Whether these effects are in fact mediated through the cannabinoid receptors has yet to be rigorously examined, however.

Anandamide can be synthesized by a variety of cell types, including neurons, RBL-2HE basophils, and J774 macrophages (Sugiura et al., 2002). It has been proposed that anandamide is rapidly inactivated by a reuptake system consisting of the anandamide membrane transporter (AMT), which transports anandamide into the cell where fatty acid amidohydrolase (FAAH) hydrolyzes anandamide into arachidonic acid and ethanolamine (Di Marzo et al., 1994). Although FAAH appears to be the primary enzyme responsible for the hydrolysis of anandamide, an acid amidase has recently been identified which is also capable of hydrolyzing anandamide (Ueda et al., 2001b). In addition to metabolism by FAAH and the acid amidase, anandamide can also be oxygenated by cyclooxygenase-2 into prostaglandin E_2 ethanolamide and perhaps other cyclooxygenase products as well (Yu et al., 1997). Prostaglandin E_2-ethanolamide appears to mediate physiological effects similar to those produced by prostaglandin E_2 (Ross et al., 2002).

Anandamide is well known to cause a number of immunomodulatory effects including modulation of cytokine production, but nothing to date has been published concerning the effects of anandamide upon IL-2 secretion. Moreover, previously published studies from this laboratory have shown that, 2-arachidonyl glycerol, a

compound that is closely related to anandamide structurally, causes inhibition of interleukin-2 production in activated T cells (Ouyang et al., 1998). The objective of the present studies was then to determine the effect of anandamide upon interleukin-2 secretion. Interleukin-2 is an autocrine/paracrine factor secreted by activated T cells, which promotes T cell proliferation. Interleukin-2 has virtually no basal level of expression, but is rapidly secreted upon T cell stimulation such that it is a hallmark of T cell activation. The current studies demonstrate that anandamide causes a concentration-dependent inhibition of interleukin-2 secretion in primary splenocytes, which is independent of CB1/CB2 and involves the activation of PPARγ by a cyclooxygenase-2 metabolite of anandamide.

Methods

Reagents – Anandamide, SR141716A and SR144528 were provided by the National Institute of Drug Abuse. UCM707, MAFP, piroxicam, NS398, and T0070907 were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Animals and cell cultures – Female B6C3F1 mice, 6 weeks of age, were purchased from Charles River (Dortage, MI). On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (5 animals/cage), and quarantined for 1 week. Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum*. Mice were not used for experimentation until their body weight was 17-20 g. Animal holding rooms were kept at 21-24ºC and 40-60% relative humidity with a 12 h light/dark cycle. Spleens were isolated aseptically and made into single-cell suspensions (1×10^6 c/ml). Cells were cultured in RPMI-1640 supplemented with 100 units penicillin/ml, 100 units streptomycin/ml, 50 µM 2-mercapoethanol, and 2% bovine calf serum.

Interleukin-2 protein quantification – Splenocytes were cultured in triplicate (1×10^6 c/ml) in 48-well culture plates (800 µL/well). The cells were pretreated with antagonist or vehicle (or not pretreated in the case of the anandamide and arachidonic acid concentration responses) for 30 min prior to treatment with either anandamide or arachidonic acid. Splenocytes were activated 30 min later with 40 nM PMA and 0.5 µM ionomycin. The supernatants were collected 24 h after stimulation and interleukin-2

protein was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) method previously described (Faubert Kaplan et al., 2003). The interleukin-2 standard (mouse recombinant interleukin-2), purified rat anti-mouse interleukin-2 antibody, and biotinylated anti-mouse interleukin-2 antibody were purchased from Pharmingen (San Diego, CA).

Statistical Analysis – The mean ± SE was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance (ANOVA). For those experiments with two factors, a 2-way ANOVA was employed. Dunnett's two-tailed t test was used to compare treatment groups to the vehicle control when significant differences were observed (Dunnett, 1955). IC_{50} values were calculated from the average of four different concentration responses using Prism Graphpad software.

Results

Anandamide causes a robust inhibition of interleukin-2 secretion, which is unaffected by pretreatment with the CB1/CB2 antagonists. Previous studies from our laboratory have shown that the endogenous cannabinoid, 2-AG, produced a concentration-dependent inhibition of interleukin-2 in activated T cells (Ouyang et al., 1998). In light of these findings, a similar analysis of anandamide was performed. In splenocytes activated with PMA and ionomycin, anandamide caused a concentration-dependent inhibition of interleukin-2 with an IC_{50} of 11.4 μM (Fig. 1). The subsequent experiments were designed to determine the mechanism by which anandamide causes inhibition of interleukin-2 with the initial studies focusing upon the cannabinoid receptors. Although CB2 is the predominant cannabinoid receptor expressed in immune cells, low levels of CB1 transcripts have been detected in many immune cells, including T cells (Galiegue et al., 1995). Therefore, the role of both CB1 and CB2 on the inhibition of interleukin-2 secretion by anandamide was evaluated. Pretreatment of primary splenocytes with the CB1 and CB2 antagonists, SR141716A and SR144528, used in combination (0.05/0.05, 0.5/0.5, and 5/5 μM), prior to treatment with anandamide (10 μM) did not attenuate anandamide-mediated inhibition of interleukin-2 secretion (Fig. 2). At the highest concentration used (5/5 μM), SR141716A and SR144528 when employed in combination in the absence of anandamide, caused inhibition of interleukin-2 secretion. Additionally, neither pretreatment with SR141716A nor SR144528 alone attenuated anandamide-mediated suppression of interleukin-2 secretion (data not shown).

Arachidonic acid also causes a concentration-dependent inhibition of interleukin-2 secretion. Because it was unclear whether the inhibitory effect of anandamide upon interleukin-2 secretion was due to the parent molecule or a hydrolysis product of anandamide, the effect of arachidonic acid upon interleukin-2 secretion was also evaluated. Treatment of primary splenocytes with various concentrations of arachidonic acid (0.1 – 20 µM) caused a concentration-dependent inhibition of interleukin-2 secretion (Fig. 3). The magnitude of interleukin-2 inhibition produced by arachidonic acid was very similar to that observed with anandamide as evidenced by the calculated IC_{50} values: 11.4 µM and 10.3 µM for anandamide and arachidonic acid, respectively.

Neither inhibition of the AMT nor inhibition of FAAH attenuates suppression of interleukin-2 by anandamide. Because arachidonic acid produced a concentration-responsive suppression of interleukin-2 secretion with a profile of inhibition which resembled that of anandamide, it seemed reasonable that inhibition of interleukin-2 by anandamide was mediated by arachidonic acid, which is likely formed from the hydrolysis of anandamide, rather than by the parent molecule of anandamide itself. It has been postulated that the AMT may be coupled to FAAH, the chief enzyme responsible for anandamide hydrolysis and that these two proteins represent a major mechanism for the uptake and catabolism of anandamide (Giuffrida et al., 2001). As such, the potential roles of the AMT and FAAH in anandamide-mediated interleukin-2 inhibition were studied. Pretreatment with the AMT inhibitor, UCM707, at various concentrations (1 - 20 µM), did not attenuate anandamide-mediated suppression of interleukin-2 secretion (Fig. 4). Prior to evaluation of the effect of the FAAH inhibitor,

MAFP, upon anandamide-mediated interleukin-2 inhibition, the effect of MAFP upon interleukin-2 in the absence of anandamide was determined. MAFP caused a concentration-responsive inhibition of interleukin-2 secretion at concentrations of 5 μM and higher (data not shown). Because MAFP is also a PLA_2 inhibitor and previous studies from this laboratory have shown that PLA_2 inhibitors cause a decrease in interleukin-2 production, the inhibitory effect of MAFP upon interleukin-2 secretion was not unexpected (Ouyang and Kaminski, 1999). Based upon the aforementioned study, 1 μM MAFP was the maximum concentration used for subsequent experiments due to the absence of an effect upon interleukin-2 coupled with the fact that the concentration is sufficient to inhibit FAAH (IC_{50} = 2.5 nM). Pretreatment with a broad range of concentrations of MAFP (0.001 – 1 μM) did not attenuate the inhibition of IL-2 secretion by anandamide (Fig. 5).

Suppression of interleukin-2 secretion by anandamide and arachidonic acid is attenuated by the nonspecific cyclooxygenase inhibitors, flurbiprofen and piroxicam. Due to the similarity in the concentration responses of anandamide and arachidonic acid in the inhibition of interleukin-2, the role of the cyclooxygenase enzymes was investigated. Initial evaluation of concentration responses of flurbiprofen determined 50 μM to be the optimum concentration for inhibition of cyclooxygenase-1 and cyclooxygenase-2 in splenocytes (data not shown). The higher concentration of 100 μM flurbiprofen caused marked inhibition of interleukin-2 by itself, which is not surprising since flurbiprofen (in the range of 100 – 1000 μM) is known to inhibit the activation of nuclear factor κ of B cells, an important transcription factor for interleukin-2 transcription

(Tegeder et al., 2001). Although 50 µM flurbiprofen caused inhibition of interleukin-2 secretion in the absence of anandamide or arachidonic acid, the inhibition was far less robust than that which was caused by 100 µM flurbiprofen and nonetheless still caused an almost complete reversal of anandamide-mediated interleukin-2 inhibition (Fig. 6a). Likewise, flurbiprofen pretreatment also significantly attenuated the suppression of interleukin-2 mediated by arachidonic acid (Fig. 6b). Similar to flurbiprofen, pretreatment of primary splenocytes with piroxicam partially attenuated the inhibitory activity of both anandamide as well as arachidonic acid upon interleukin-2 secretion (Fig. 7). Moreover, the effect of piroxicam upon anandamide-mediated suppression of interleukin-2 is concentration-dependent (Fig. 9a).

NS398, a cyclooxygenase-2 specific inhibitor, partially attenuated the inhibition of IL-2 secretion by anandamide and arachidonic acid. In order to determine whether the inhibitory activity of anandamide and arachidonic acid upon interleukin-2 was mediated by a cyclooxygenase-1 or cyclooxygenase-2 metabolite, the ability of NS398, a cyclooxygenase-2 specific inhibitor, to attenuate suppression of interleukin-2 by anandamide and arachidonic acid was evaluated. Similar to flurbiprofen and piroxicam, NS398 also attenuated the inhibition of IL-2 secretion by anandamide and arachidonic acid (Fig. 8). In addition, the attenuation by NS398 of anandamide-mediated inhibition of IL-2 secretion is also concentration-dependent (Fig. 9b). Conversely, the cyclooxygenase-1 specific inhibitors, SC560 and FR122047, did not have an effect upon the suppression of interleukin-2 secretion by anandamide (data not shown).

Suppression of interleukin-2 secretion by anandamide is partially antagonized by pretreatment with T0070907, a PPARγ antagonist. Because activated PPARγ has been shown to cause inhibition of interleukin-2 secretion and a number of cyclooxygenase products, such as PGD_2 and 15-deoxy-$\Delta^{12,14}$ PGJ_2, have been shown to be endogenous agonists of PPARγ; the ability of T0070907, a specific PPARγ antagonist, to antagonize inhibition of interleukin-2 secretion by anandamide was examined. Pretreatment of primary splenocytes with T0070907, at various concentrations (0.1 – 10 μM) caused a concentration-dependent antagonism of anandamide-mediated suppression of interleukin-2 secretion. Although the highest concentration of T0070907 used (10 μM) caused inhibition of interleukin-2 in the absence of anandamide, T0070907 nevertheless still caused a significant reversal of the effects of anandamide upon interleukin-2 at this concentration.

Discussion

The present studies demonstrate that anandamide causes a concentration-dependent inhibition of interleukin-2 secretion. The current studies also show that suppression of interleukin-2 secretion mediated by anandamide is not attenuated by pretreatment with the cannabinoid receptor antagonists, SR141716A and SR144528, suggesting that it is independent of both CB1 and CB2. Interestingly, arachidonic acid also caused a concentration-responsive inhibition of interleukin-2, which is similar to that of anandamide. The similarity was also evident from the calculated IC_{50} values, which were 11.4 μM and 10.3 μM for anandamide and arachidonic acid, respectively. Although the suppression of interleukin-2 by arachidonic acid suggests that inhibition of interleukin-2 by anandamide may be mediated by a product of anandamide hydrolysis rather than the parent molecule itself, neither pretreatment with an AMT inhibitor nor a FAAH inhibitor attenuated suppression of interleukin-2 secretion by anandamide. Pretreatment with two different nonspecific cyclooxygenase inhibitors, flurbiprofen and piroxicam, partially attenuated inhibition of interleukin-2 secretion by both anandamide and arachidonic acid, suggesting a role for the cyclooxygenase enzymes. Likewise, NS398, a cyclooxygenase-2 specific inhibitor also attenuated the inhibition of interleukin-2 secretion by both anandamide and arachidonic acid, while the cyclooxygenase-1 specific inhibitors, SC560 and FR122047, had no effect (data not shown). In addition, T0070907, a PPARγ specific antagonist, caused a concentration-dependent partial antagonism of anandamide-mediated inhibition of interleukin-2. Collectively, the aforementioned observations suggest that the inhibition of interleukin-2

secretion is partially mediated by PPARγ, which is activated by a cyclooxygenase-2 metabolite of anandamide.

The concentrations of anandamide, which cause inhibition of interleukin-2 secretion, are in the low micromolar range. While concentrations of anandamide have been detected in the nanomolar range in rat and human plasma, the local concentrations of anandamide at the various target sites have yet to be determined. Because anandamide is synthesized de novo from membrane components, it seems likely, however, that concentrations of anandamide might be quite high within the microcosms of the target regions. Measurements of anandamide are further confounded by the lability of the compound. Moreover, anandamide has generally been measured in normal healthy tissue and plasma samples, whereas these levels may differ substantially in inflamed or damaged tissues. Arachidonic acid levels, for instance, have been shown to increase into the upper micromolar range in damaged tissues (Patel et al., 2003). Furthermore, arachidonic acid mobilization has been linked to increased anandamide synthesis (Pestonjamasp and Burstein, 1998).

Although there have been a number of effects of endocannabinoids attributed to CB1/CB2, there are also a growing number of reports of endocannabinoid activities which are independent of CB1/CB2 in keeping with the studies reported here (Smart et al., 2000, Ross et al., 2002). One potential mechanism for cannabinoid receptor-independent activity is the vanilloid receptor, VR1. VR1 is a ligand-gated cation channel, which is activated by heat or capsaicin. Anandamide is a full agonist of VR1 albeit at concentrations 10 to 20 times higher than those at which it activates CB1 (Smart et al., 2000). It is notable that the VR1 agonist, capsaicin, does not cause

inhibition of interleukin-2 secretion and that the VR1 antagonist, capsazepine, does not attenuate anandamide-mediated interleukin-2 inhibition (data not shown). The current study is unique in that it not only demonstrates anandamide-mediated activity which is independent of CB1/CB2 and VR1, but also in that it shows that the metabolism of anandamide does not always lead to the cessation of physiological effects, but in certain circumstances may actually be responsible for the biological activity observed.

There have been a number of reports that anandamide mediates arachidonic acid release from various cell types, including human peripheral blood mononuclear cells (Berdyshev et al., 1997; Di Marzo et al., 1997). Therefore, an alternative explanation for the observations of the current investigation is that anandamide causes release of arachidonic acid from the primary splenocytes. The released arachidonic acid is then subsequently metabolized into an eicosanoid, which mediates the inhibition of interleukin-2 secretion. While the current studies do not negate the possibility that anandamide causes arachidonic acid release, the striking similarity in the concentration responses of anandamide and arachidonic acid suggests that the same moiety is responsible for the activity of both anandamide and arachidonic acid. Further studies will be needed to determine whether this is the case, however.

The primary mechanism, which has been identified, for the hydrolysis of anandamide is through the enzyme, FAAH. FAAH has been detected in a variety of different tissues with the highest levels found in the brain, liver, small intestine, and testes, whereas relatively low levels have been found in the spleen (Bisogno et al., 2002). Although FAAH seems to be the primary mechanism for the hydrolysis of anandamide, at least one other enzyme has been identified which hydrolyzes

anandamide. Unlike FAAH, the other amidase has an optimal pH of 5, is not inhibited by MAFP, and is also reported to have a high level of activity in the spleen (Ueda et al., 2001a). Under the conditions used for the current studies, it is likely that anandamide is either hydrolyzed by the acid amidase, another amidase which has yet to be identified, or is directly metabolized by cyclooxygenase-2.

While the vast majority of research on anandamide metabolism has focused upon FAAH, there has also been considerable interest in the role of the cyclooxygenase enzymes. There are two isoforms of the cyclooxygenase enzyme, cyclooxygenase-1 and cyclooxygenase-2. Cyclooxygenase-1 is constitutively expressed in most cell types, whereas cyclooxygenase-2 expression is induced in response to a stimulus only in certain cell types (Parente and Perretti, 2003). Both cyclooxygenase enzymes have been found in most cells within the immune system, including T cells (Tilley et al., 2001). It has been reported that anandamide can bind directly to cyclooxygenase-2 and be metabolized into prostaglandin E_2-ethanolamide and potentially other products as well (Yu et al., 1997). The same studies have also demonstrated, however, that anandamide neither binds directly to, nor is oxygenated by cyclooxygenase-1.

There is evidence to support that a number of different prostanoids can cause inhibition of interleukin-2 secretion, including prostaglandin E_2, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, and prostaglandin I_2 (Marcinkiewicz and Chain, 1993; Harris et al., 2002; Yang et al., 2000). Of the aforementioned eicosanoids, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) is the most recent to be recognized to suppress interleukin-2 secretion. There has been growing interest in 15d-PGJ_2 in a number of different research areas due to its identification as one of the most potent endogenous

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ligands of peroxisome proliferator activated receptor gamma (PPARγ). Structurally related to the hormone receptors, PPARs are also members of the nuclear receptor superfamily. Currently, there are three subtypes of PPARs, which have been identified: PPARα, PPARδ, and PPARγ. While the function of PPARδ remains unclear, PPARα and PPARγ have been best been characterized for their roles in lipid metabolism and have only recently been discovered to also be involved with immunoregulation (Clark, 2002). Although both PPARα and PPARγ are expressed in T cells, only activated PPARγ causes an inhibition of IL-2 secretion. Physical association of activated PPARγ with the transcription factor, NFAT, appears to suppress NFAT binding to the IL-2 promoter, which is thought to be the mechanism for the decrease in interleukin-2 production by PPARγ agonists (Yang et al., 2000). Similarly, activated PPARγ has also been shown to cause decreased cytokine production in activated macrophages through direct protein-protein interaction with NFκB, a key transcription factor for a number of different cytokines (Ricote et al., 1998).

15d-PGJ_2 is produced through the sequential metabolism of arachidonic acid by cyclooxygenase and PGD synthase, followed by a series of nonenzymatic transformations (Zhang and Young, 2002). Whether 15d-PGJ_2 is produced in vivo has been somewhat of a controversy, however recent studies have identified elevated levels of 15d-PGJ_2 in LPS-stimulated RAW264.7 macrophages (Shibata et al., 2002). Future studies will determine whether 15d-PGJ_2 is produced in other cell types, but it is notable that both enzymes required for 15d-PGJ_2 formation, cyclooxygenase and PGD synthase, are expressed in T cells. While 15d-PGJ_2 is one of the most potent activators of PPARγ, other cyclooxygenase products are also able to activate PPARγ. More

studies will be needed to determine exactly which cyclooxygenase products are produced from anandamide metabolism and which are subsequently responsible for the observed inhibition of interleukin-2 production.

While several studies have emerged which suggest that under certain conditions, activated PPARγ may cause apoptosis in activated T cells; there are also a number of studies suggesting that PPARγ activation may be protective against apoptosis or have no effect upon viability (Clark, 2002, Wang et al., 2002). While the cause of the differential effects of activated PPARγ upon T cell viability is unclear, it is likely that the concentration of 15d-PGJ_2, the kinetics of cell treatment, and/or the state/mode of activation may be factors. Under the specific conditions used in the present studies, there was no effect upon cellular viability. It should be noted, however, that anandamide and arachidonic acid are likely to be metabolized into a number of different products, some of which may be protective against apoptosis.

While in the majority of cases studied thus far the metabolism of anandamide has been associated with the cessation of its physiological activity, the present studies are significant because they show that metabolism of anandamide in certain cases may yield a biologically active product. It has been demonstrated here that the metabolism of anandamide by cyclooxygenase 2 yields a product, which causes inhibition of interleukin-2 secretion through activation of PPARγ.

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Footnotes

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Figure legends

Figure 1. Effect of anandamide upon PMA/Ionomycin-stimulated interleukin-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cell/ml) were treated with 0.01-20 µM of anandamide (AEA) or VH (0.1% ethanol) for 30 min followed by activation of the cells with PMA (40 nM) and ionomycin (0.5 µM). Cells were harvested 24 h later and the supernatants were analyzed for IL-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least four separate experiments.

Figure 2. Effect of cannabinoid receptor antagonists on anandamide-mediated inhibition of PMA/ionomycin-stimulated interleukin-2 production. Splenocytes (1 x 10^6 cells/ml) were pretreated with both SR141716A and SR144528 or VH (0.1% DMSO) for 30 min followed by anandamide (AEA) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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. * $p<0.05$ compared to the matched VH group. These data are representative of at least three separate experiments.

Figure 3. Effect of arachidonic acid upon PMA/ionomycin-stimulated IL-2 production in murine primary splenocytes. Splenocytes (1 x 10^6 cells/ml) were treated with 0.01-20 µM of arachidonic acid (AA) or VH (0.1% ethanol) for 30 min followed by activation of

the cells with PMA (40 nM) and ionomycin (0.5 µM). Cells were harvested 24 h later and the supernatants were analyzed for interleukin-2 protein by ELISA. Cellular viability was ≥ 85% for all treatment groups as assessed by trypan blue exclusion. The results are the mean ± standard error of triplicate cultures. * $p<0.05$ compared to VH group. These data are representative of at least four separate experiments.

Figure 4. Effect of the anandamide membrane transporter inhibitor, UCM707, on anandamide-mediated inhibition of PMA/ionomycin-stimulated interleukin-2 production. Splenocytes (1 x 10^6 cells/ml) were pretreated with UCM707 or VH (0.1% ethanol) for 30 min followed by anandamide (AEA) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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. The groups that were treated with both UCM707 and AEA were not significantly different from the control group, VH + AEA. These data are representative of at least three experiments.

Figure 5. Effect of the FAAH inhibitor, MAFP, on anandamide-mediated inhibition of PMA/ionomycin-stimulated interleukin-2 production. Splenocytes (1 x 10^6 cells/ml) were pretreated with MAFP (1 µM) or VH (0.01% DMSO) for 30 min followed by anandamide (AEA) treatment for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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. * $p<0.05$ compared to VH + AEA group. These data are representative of at least three experiments.

Figure 6. Effect of the nonselective cyclooxygenase inhibitor, flurbiprofen, on inhibition of interleukin-2 secretion by anandamide and arachidonic acid. Splenocytes (1×10^6 cells/ml) were pretreated with 50 µM flurbiprofen (FBN) or VH (0.05% ethanol) for 30 min followed by treatment with either A.) anandamide (AEA) or B.) arachidonic acid (AA) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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 FBN + VH group. c denotes $p<0.05$ compared to the matched VH group. These data are representative of at least three experiments.

Figure 7. Effect of the cyclooxygenase-1 selective inhibitor, piroxicam, on the inhibition of IL-2 secretion by AEA and AA. Splenocytes (1×10^6 cells/ml) were pretreated with piroxicam (20 or 50 µM) or VH (0.1% ethanol) for 30 min followed by treatment with either A.) anandamide (AEA) or B.) arachidonic acid (AA) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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 piroxicam + VH group. c denotes $p<0.05$ compared to the matched VH group. These data are representative of at least three experiments.

Figure 8. Effect of the cyclooxygenase-2 specific inhibitor,NS398, on the inhibition of IL-2 secretion by AEA and AA. Splenocytes (1×10^6 cells/ml) were pretreated with NS398 (10 µM) or VH (0.02% ethanol) for 30 min followed by treatment with either A.) anandamide (AEA) or B.) arachidonic acid (AA) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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 piroxicam + VH group. c denotes $p<0.05$ compared to the matched VH group. These data are representative of at least three experiments.

Figure 9. Piroxicam and NS398 attenuate the inhibition of IL-2 secretion by anandamide in a concentration-dependent manner. Splenocytes (1×10^6 cells/ml) were pretreated with either A.) piroxicam or B.) NS398 for 30 min followed by treatment with either anandamide (AEA) or VH (0.04% ethanol) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 µM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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 VH + anandamide group. These data are representative of at least three experiments.

Figure 10. Effect of the PPARγ-specific antagonist on anandamide-mediated inhibition of PMA/ionomycin-stimulated interleukin-2 production. Splenocytes (1×10^6 cells/ml) were pretreated with T0070907 or VH (0.1% DMSO) for 30 min followed by treatment with 20 μM anandamide (AEA) for 30 min. Cells were then stimulated with PMA (40 nM) and ionomycin (0.5 μM) for 24 h. The supernatants were harvested and interleukin-2 production was measured 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. * $p<0.05$ compared to the VH + anandamide group. These data are representative of at least three separate experiments.

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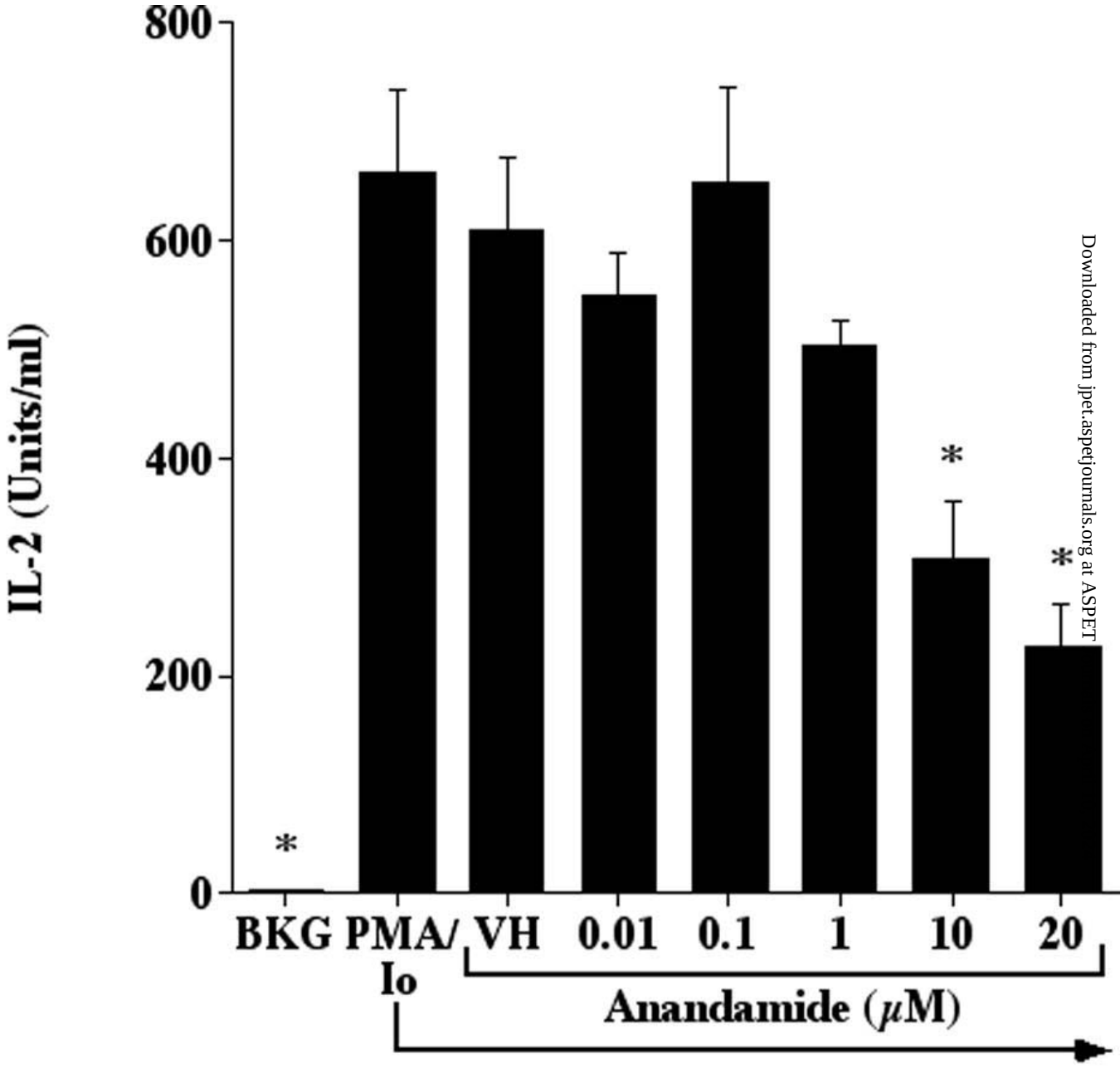

Fig. 1

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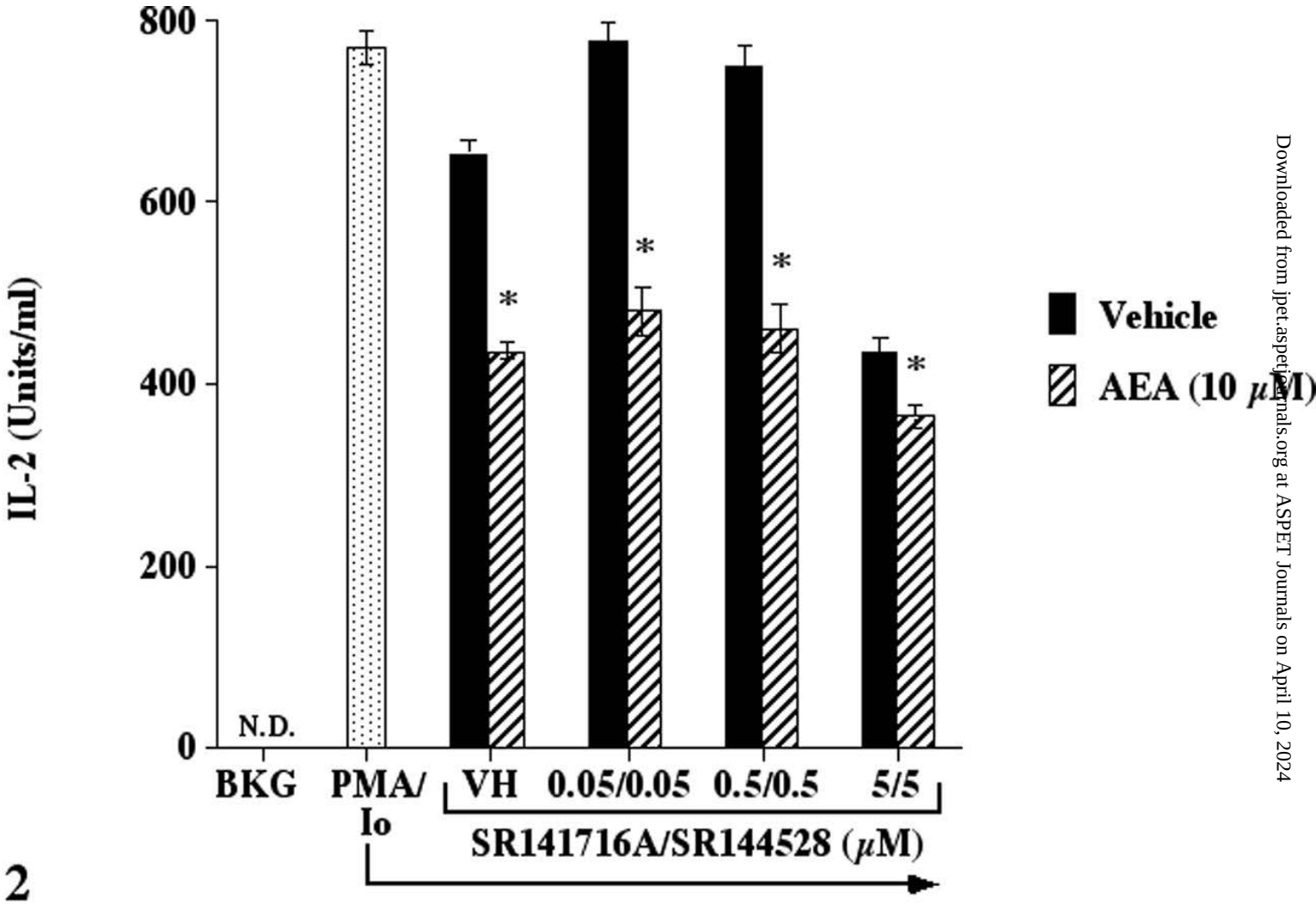

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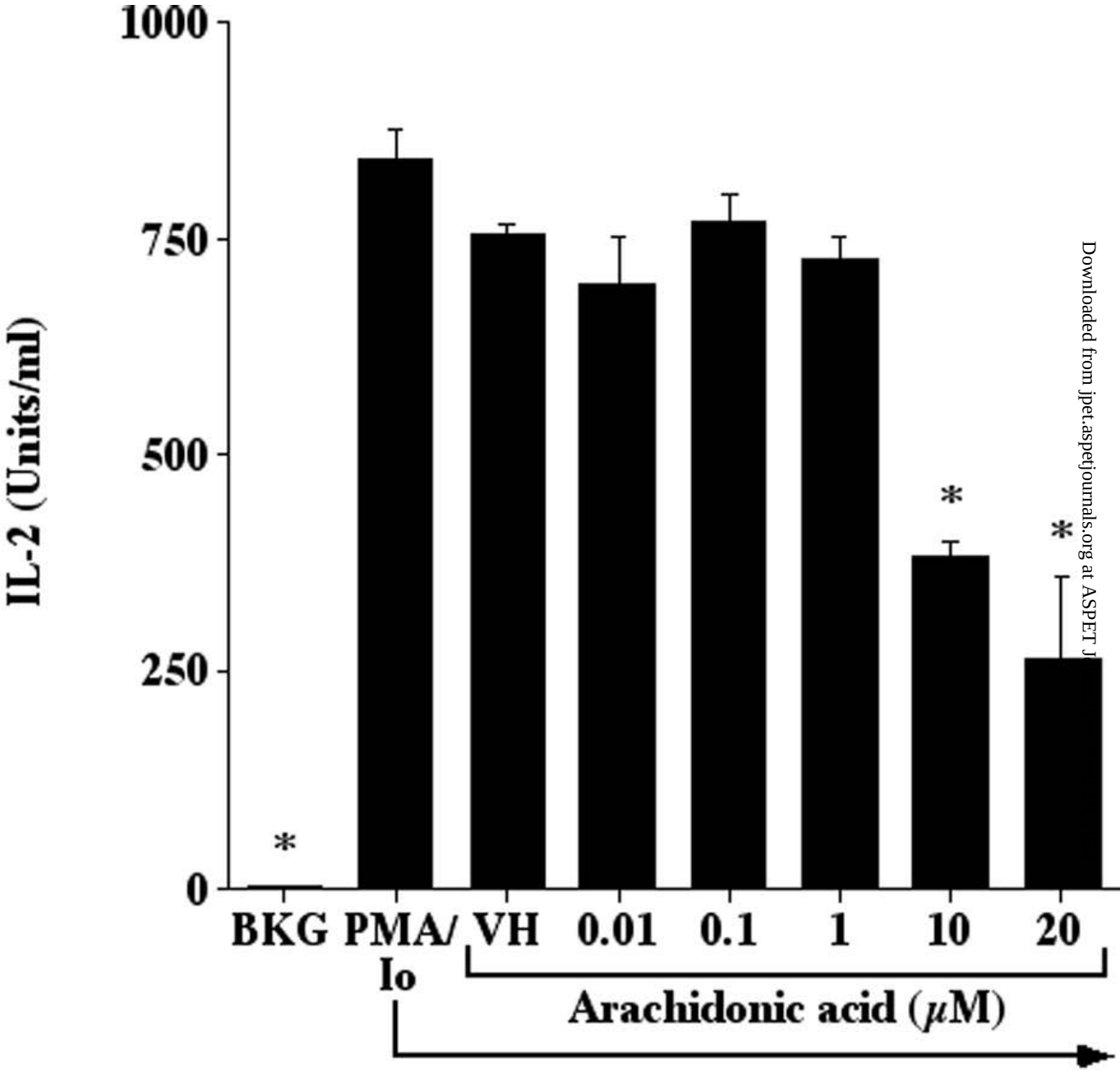

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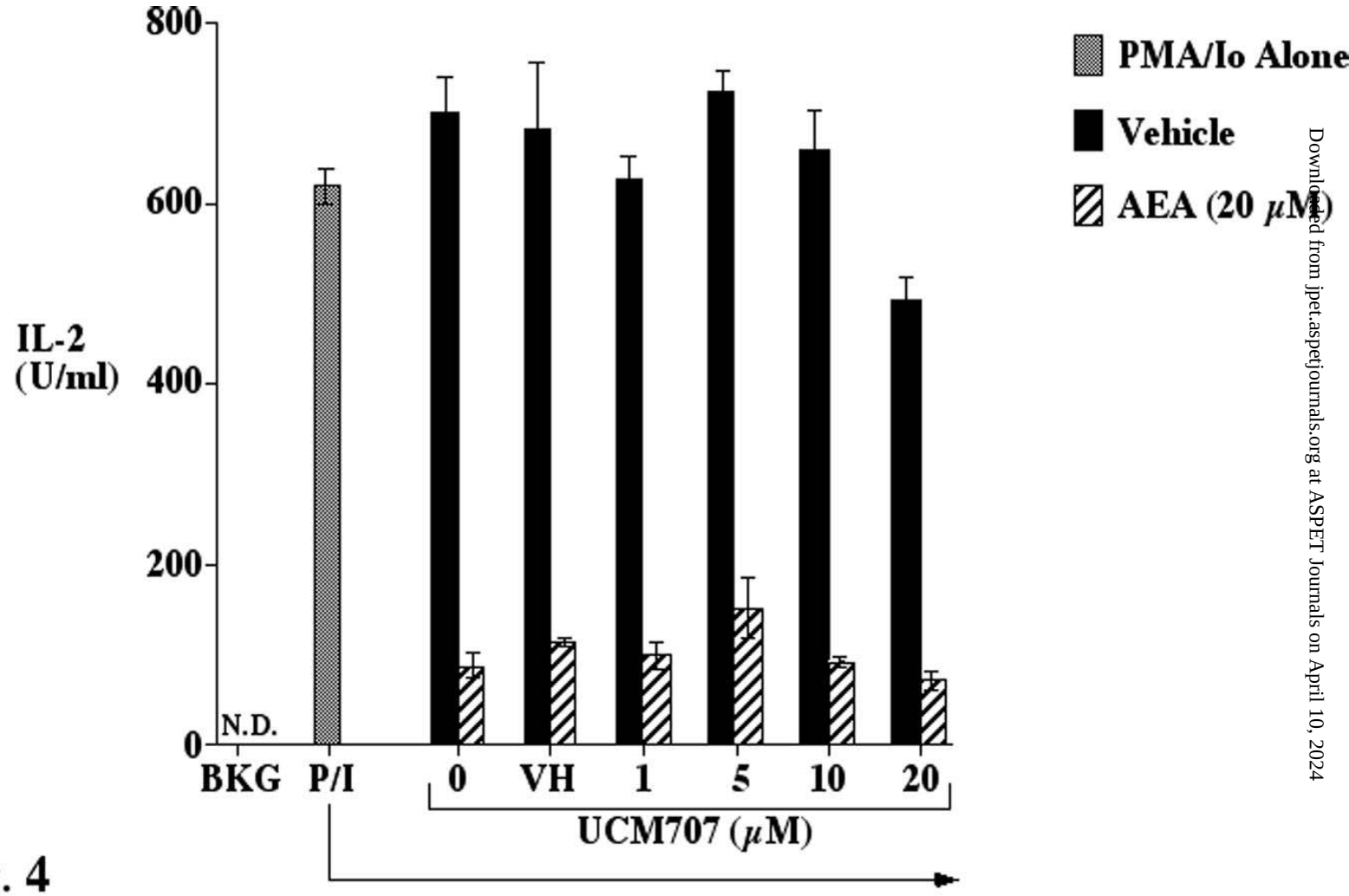

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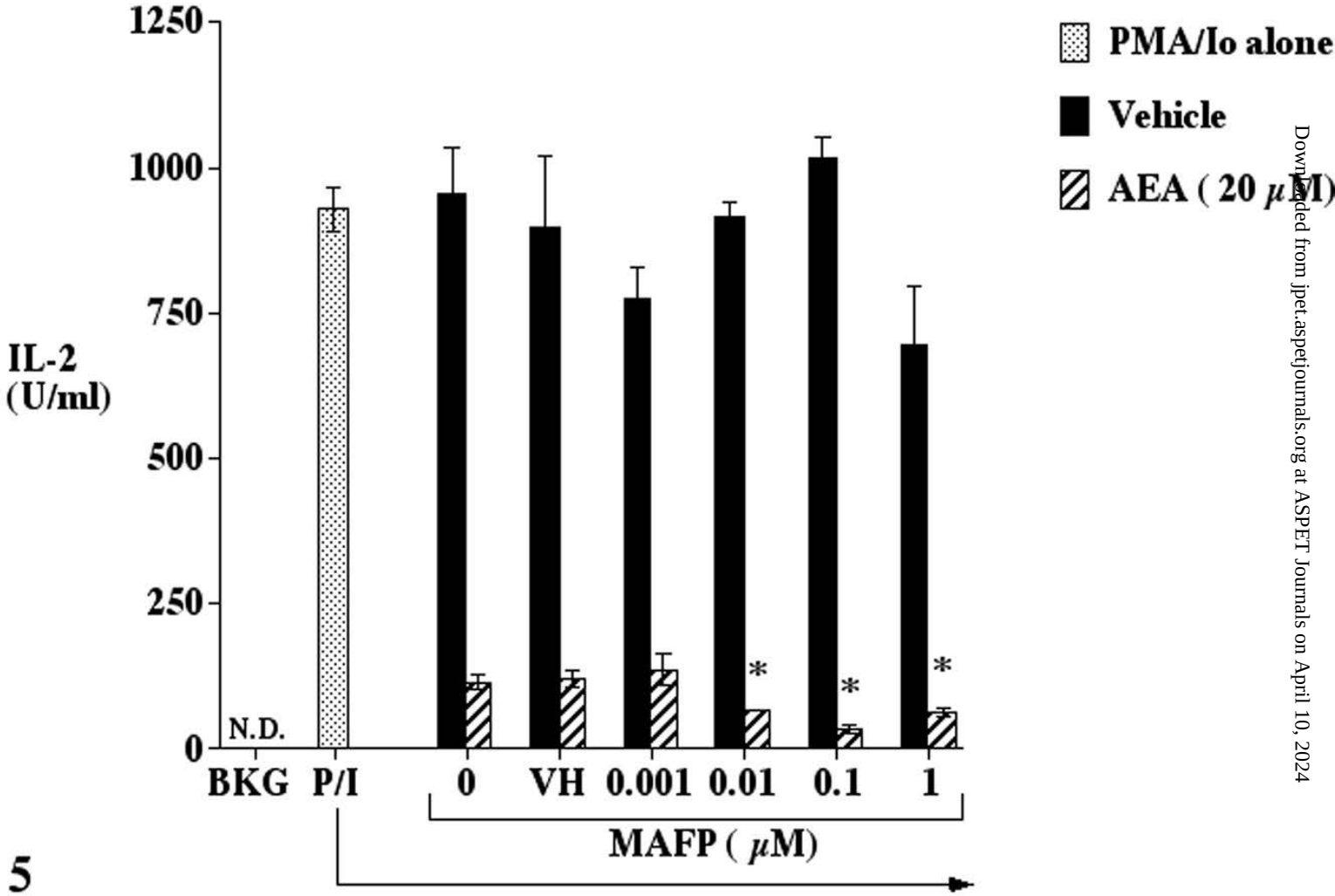

Fig. 5

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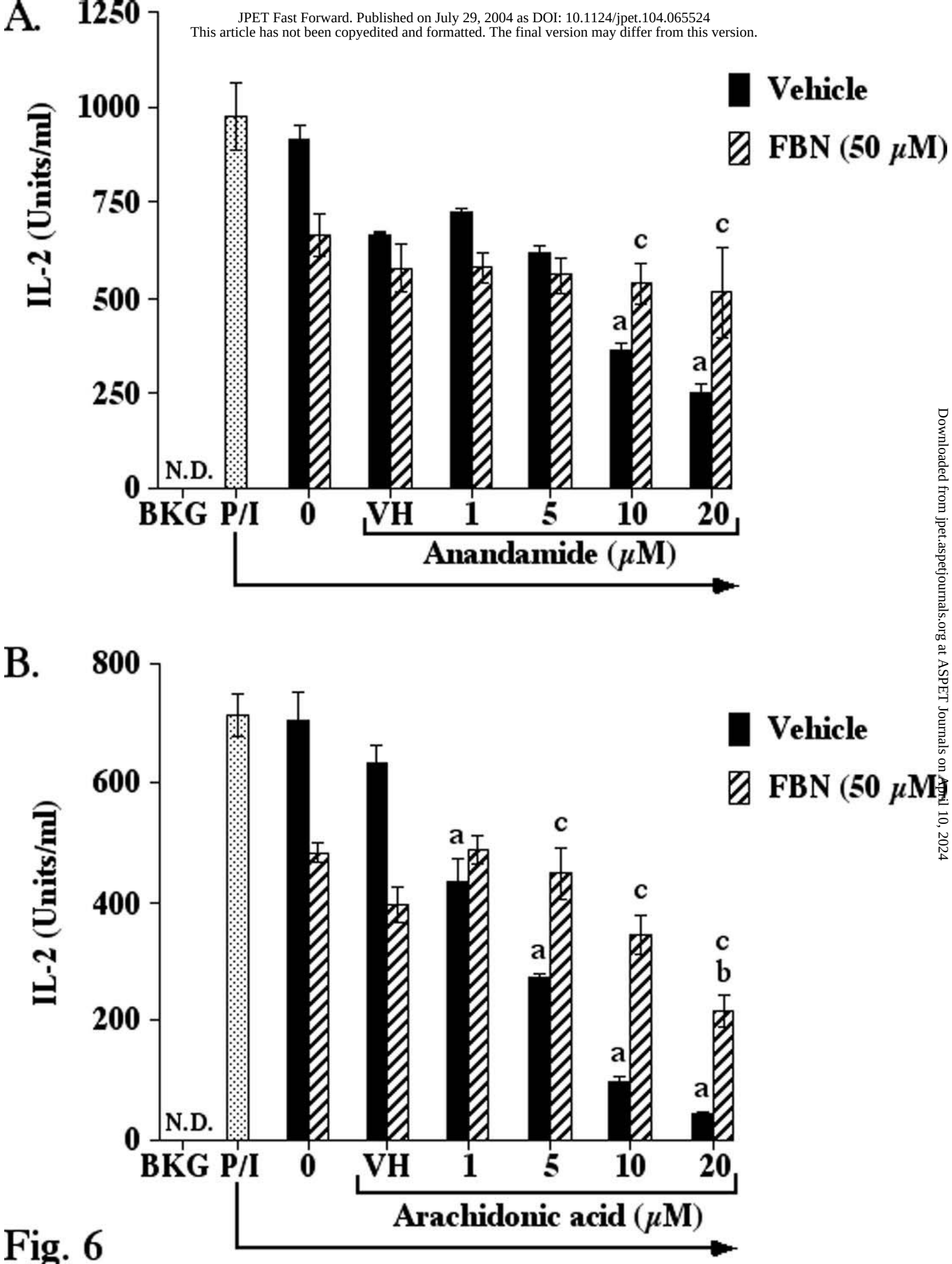

Fig. 6

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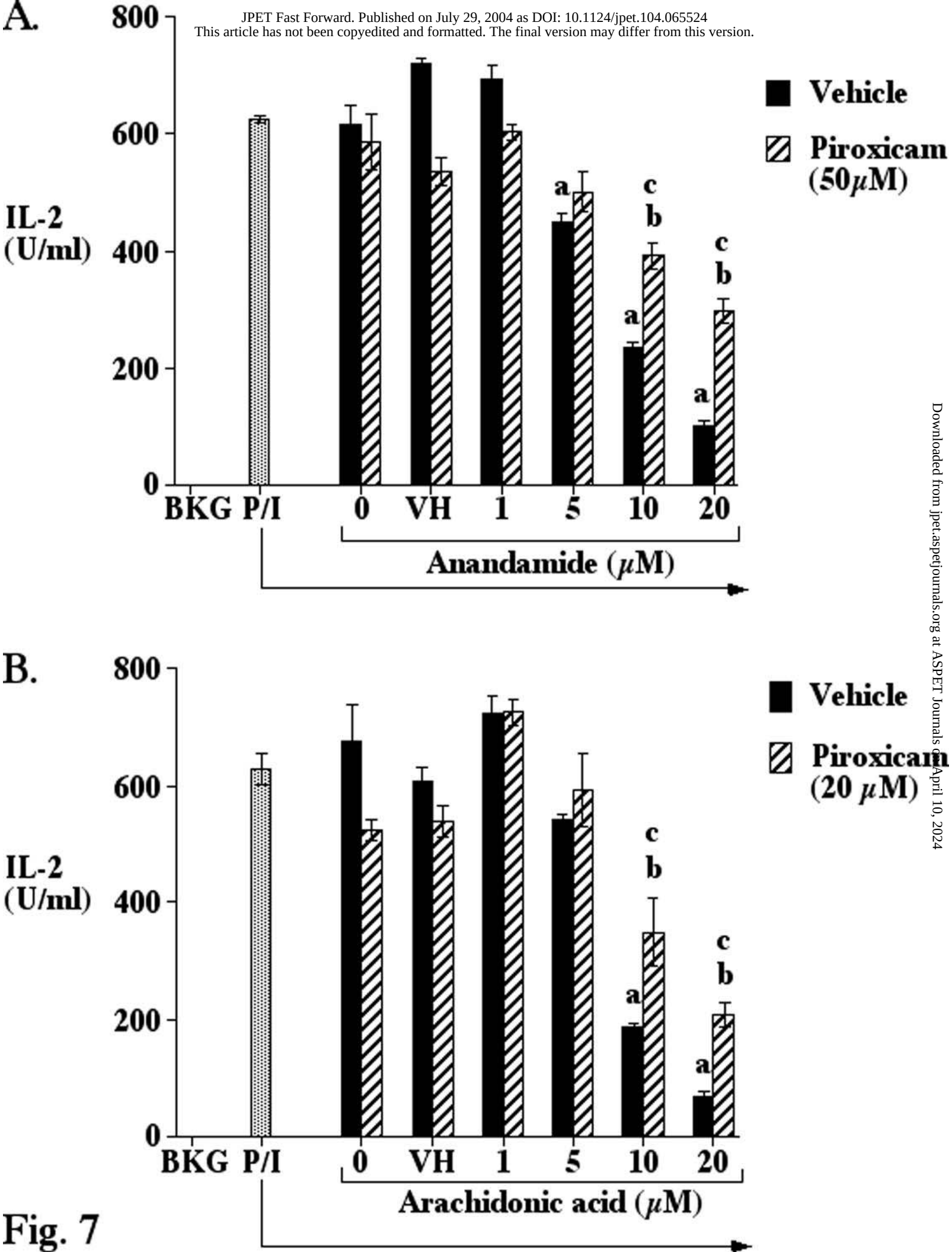

Fig. 7

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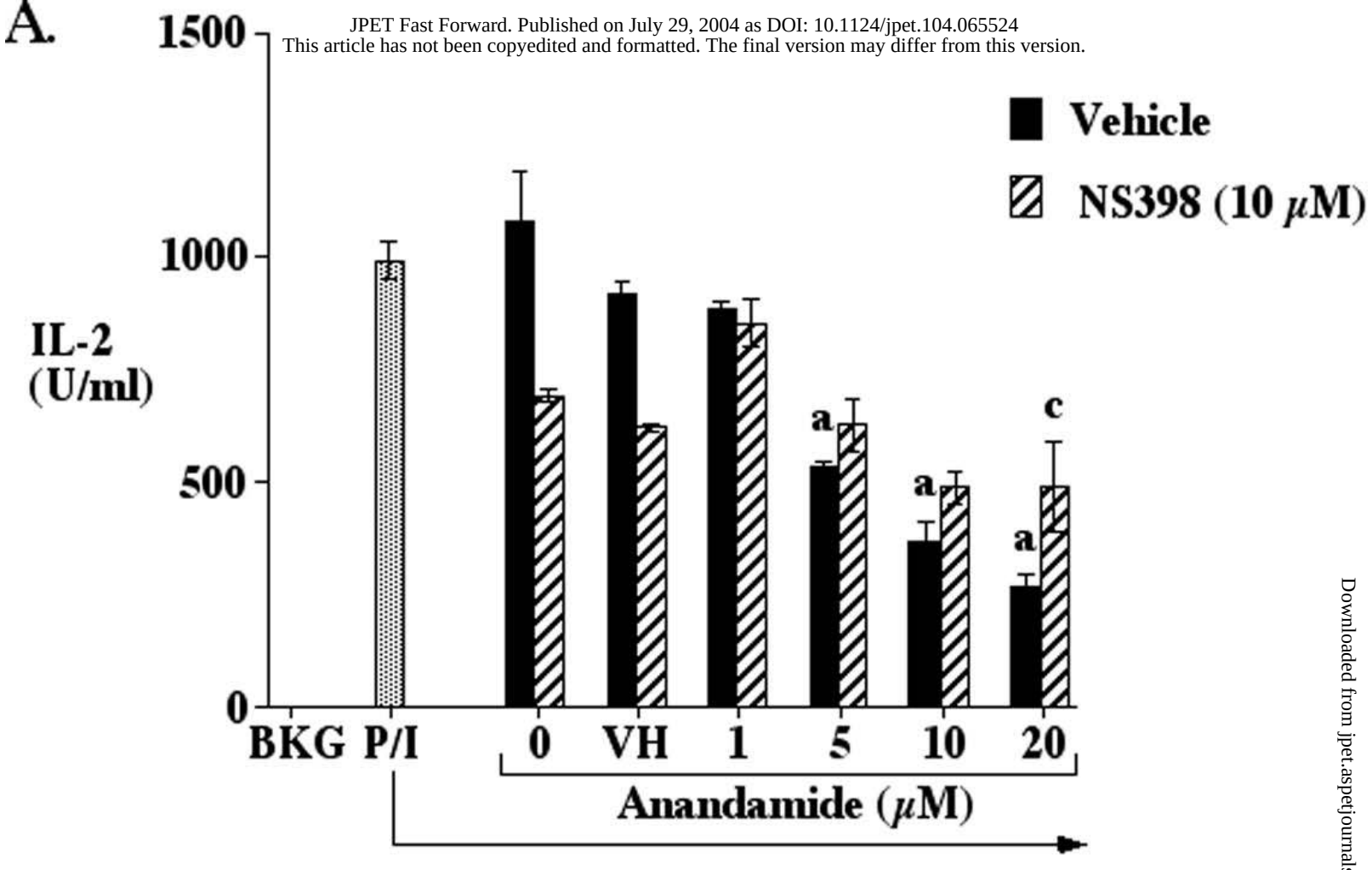

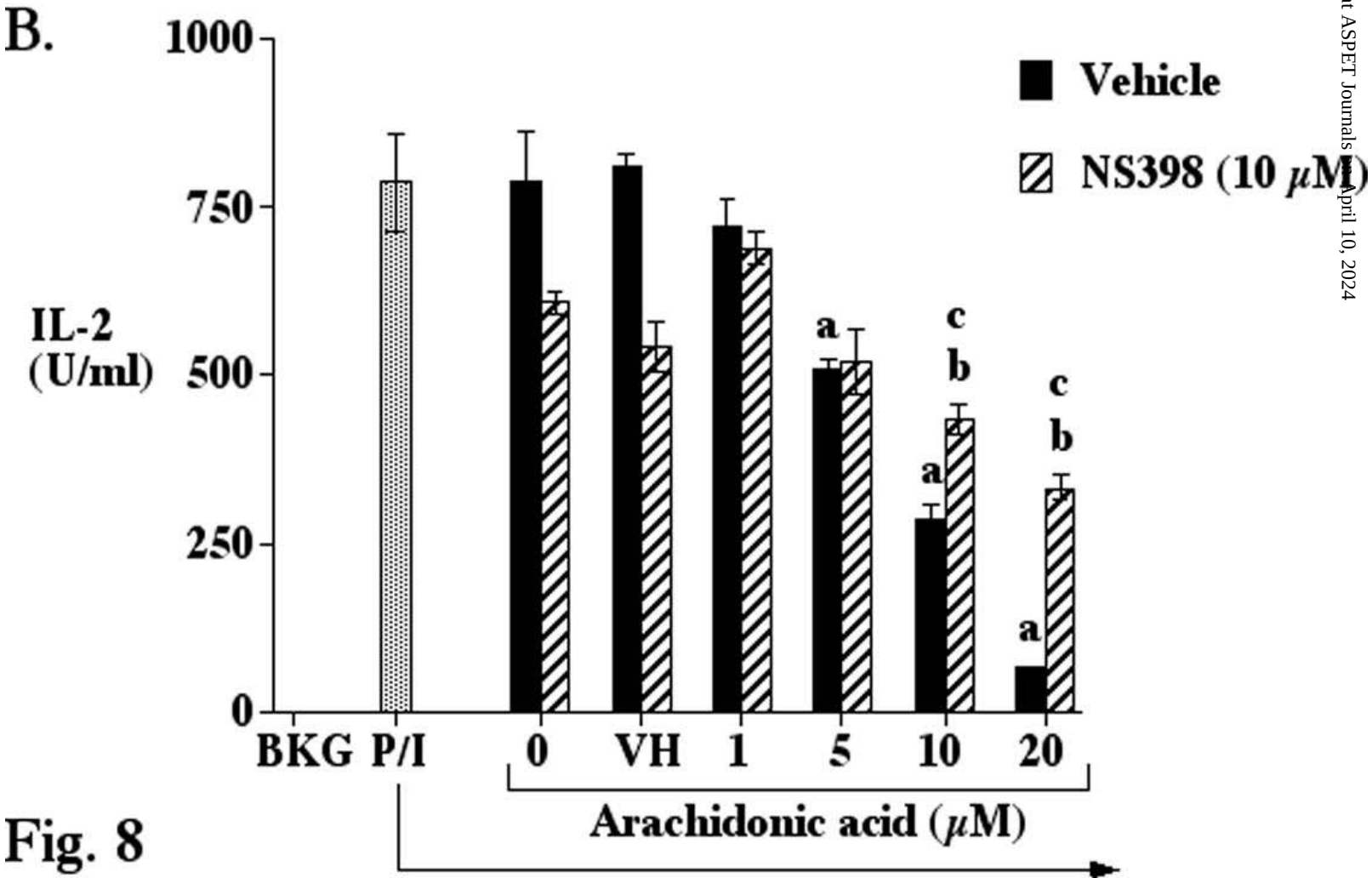

Fig. 8

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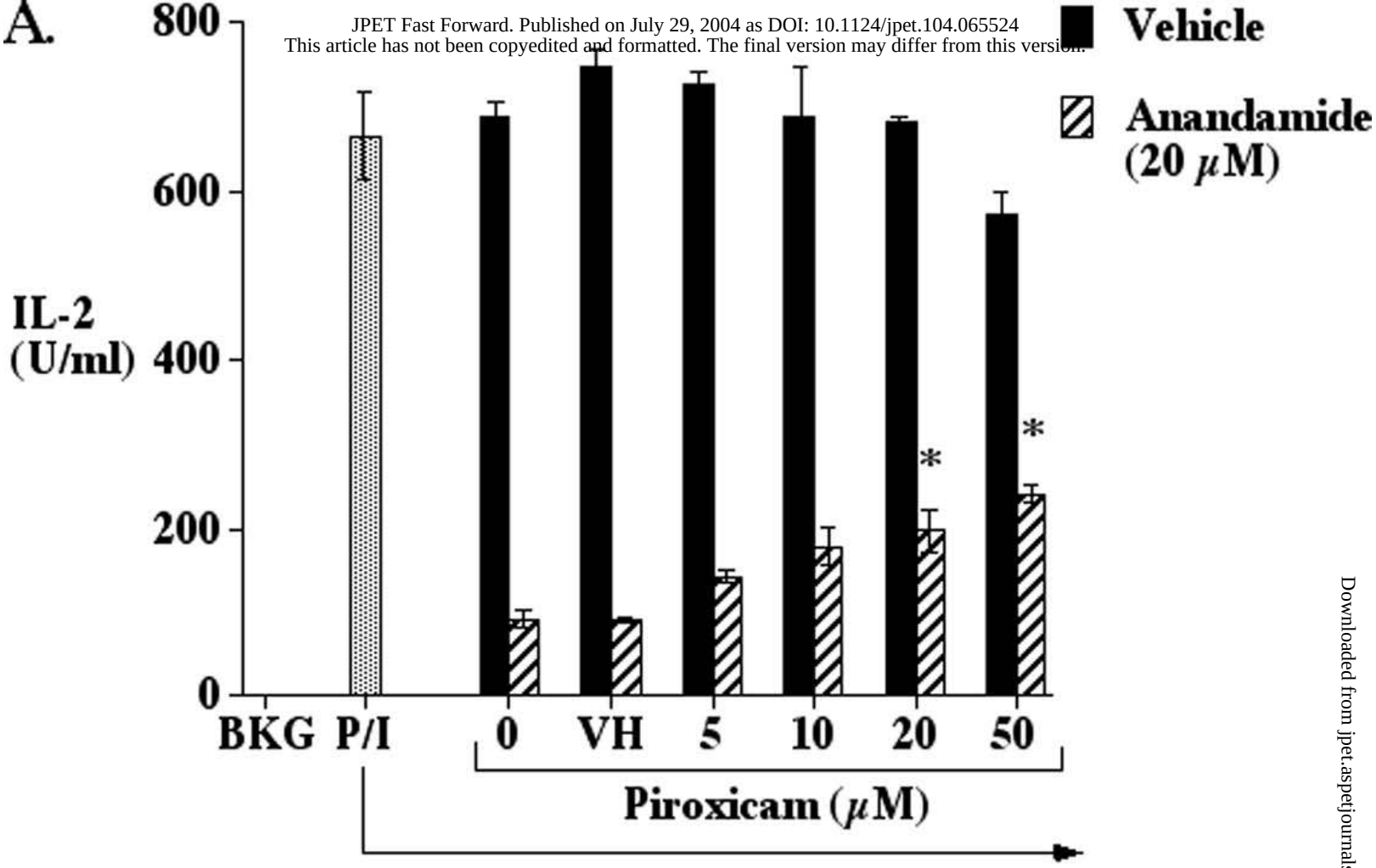

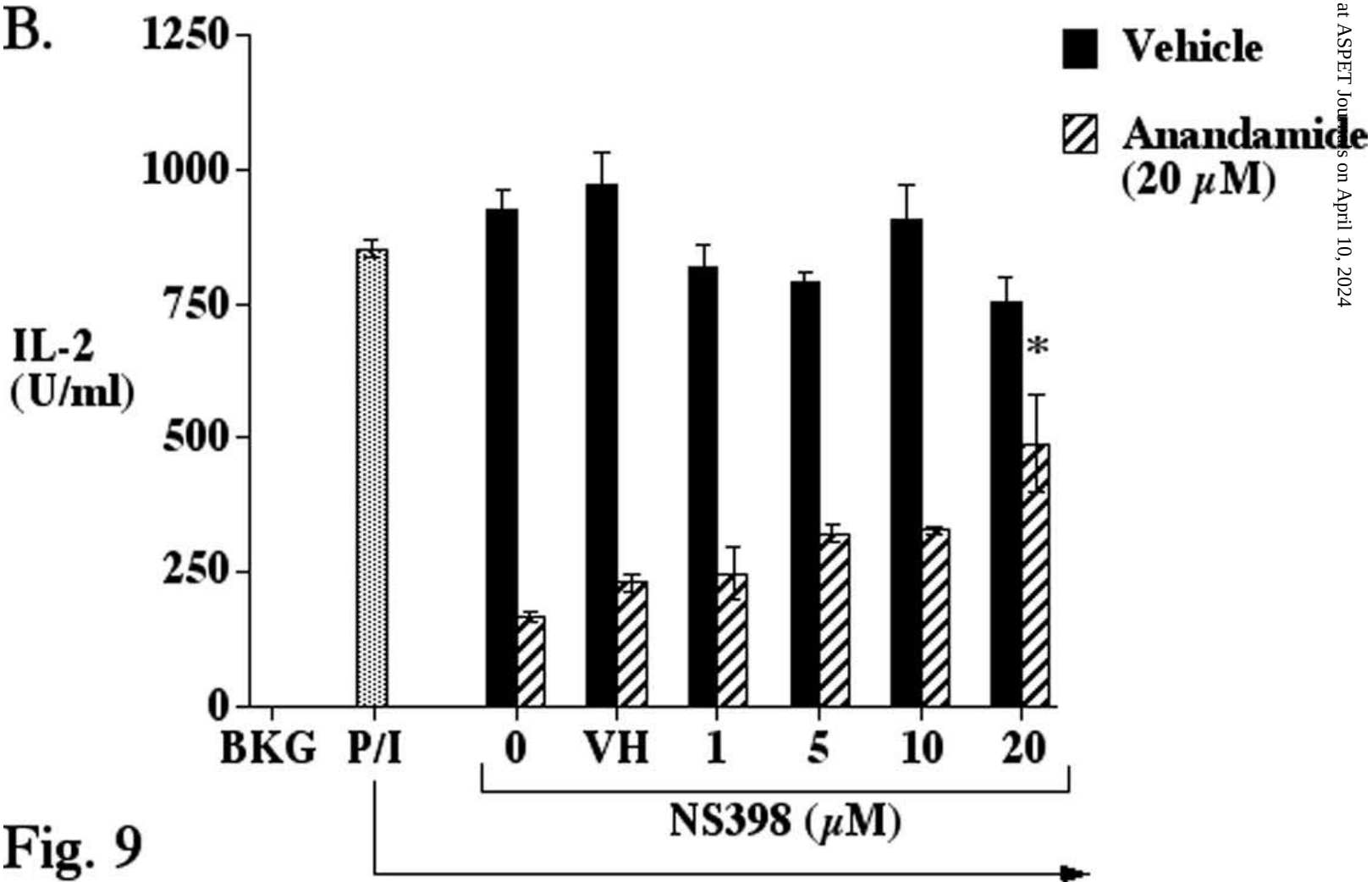

Fig. 9

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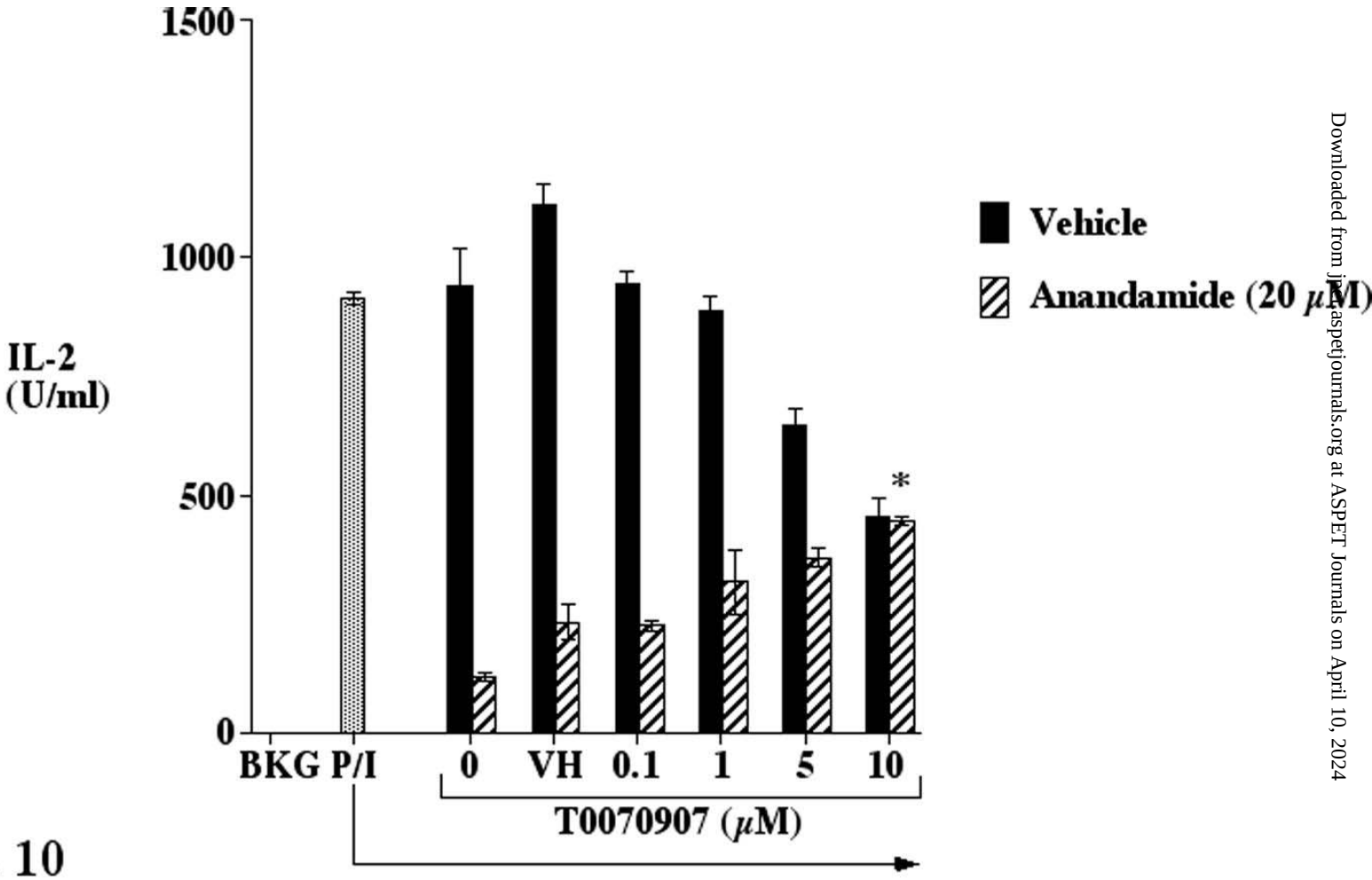

Fig. 10