Pharmacological inhibition of endocannabinoid degradation modulates the expression of inflammatory mediators in the hypothalamus following an immunological stressor

Kerr D.M.[1,2,3], Burke N.N.[1,3], Ford G.K.[2,3], Connor T.J.[4], Harhen B.[3], Egan L.J.[2], Finn D.P.[2,3] and Roche M.[1,3]*

[1]Physiology and [2]Pharmacology and Therapeutics, School of Medicine, [3]Centre for Pain Research and NCBES Neuroscience Cluster, National University of Ireland, Galway, Ireland.

[4]Neuroimmunology Research Group, Trinity College Institute of Neuroscience, Department of Physiology & School of Medicine, Trinity College, Dublin 2, Ireland

Corresponding Author:

Dr Michelle Roche, Physiology, School of Medicine, National University of Ireland, Galway, University Road, Galway, Ireland.

Tel: 353 91 495427 Fax: 353 91 494544

Email: Michelle.roche@nuigalway.ie

Abbreviations: 2-AG 2-arachidonyl glycerol; AEA anandamide; FAAH Fatty acid amide hydrolyase; HPA axis Hypothalamic-pituitary-adrenal axis; IL interleukin; LPS Lipopolysaccharide; MAGL monoacylglycerol lipase; OEA *N*-oleoyl ethanolamide; PEA *N*-palmitoyl ethanolamide; SOCS suppressor of cytokine signalling; STAT signal transducer and activation of transcription.

ABSTRACT

The endocannabinoid system is an important regulator of the nervous, neuroendocrine and immune systems, thus representing a novel therapeutic target for stress-related neuroinflammatory and psychiatric disorders. However, there is a paucity of data relating to the effects of endocannabinoids on neuroinflammatory mediators following an immune stress/challenge *in vivo*. This study investigated the effects of URB597, a selective inhibitor of fatty acid amine hydrolyase (FAAH), the enzyme that preferentially metabolises anandamide, on lipopolysaccharide (LPS)-induced increases in the expression of immune mediators in the hypothalamus. Systemic administration of URB597 increased the levels of anandamide and the related *N*-acylethanolamines, *N*-palmitoyl ethanolamide and *N*-oleoyl ethanolamide, but not 2-arachidonoyl glycerol, in the hypothalamus and spleen. URB597 attenuated the LPS-induced increase in interleukin (IL)-1β expression while concurrently augmenting the LPS-induced increase in suppressor of cytokine signalling (SOCS)-3 expression. In addition, URB597 tended to enhance and reduce the LPS-induced increase in IL-6 and IL-10 mRNA expression respectively. LPS-induced increases in peripheral cytokine levels or plasma corticosterone were not altered by URB597. The present study provides evidence for a role for FAAH in the regulation of LPS-induced expression of inflammatory mediators in the hypothalamus. Improved understanding of endocannabinoid-mediated regulation of neuroimmune function has fundamental physiological and potential therapeutic significance in the context of stress-related disorders.

Keywords: Endocannabinoid, anandamide, 2-AG, cytokine, HPA axis

The endocannabinoid system comprises the CB_1 and CB_2 receptors, the naturally occurring endogenous ligands, anandamide (AEA) and 2-arachidonyl glycerol (2-AG); and the enzymes involved in their synthesis and degradation. The enzyme fatty acid amide hydrolyase (FAAH) preferentially metabolises AEA (Cravatt et al., 1996a) and although 2-AG also acts as a substrate for FAAH, monoacylglycerol lipase (MAGL) is considered the primary enzyme involved in 2-AG inactivation (Dinh et al., 2002; Long et al., 2009). By competing with AEA for the catalytic site of FAAH, fatty acid amides such as the *N*-acylethanolamines, *N*-palmitoyl ethanolamide (PEA) and *N*-oleoyl ethanolamide (OEA) are capable of enhancing endocannabinoid signalling (Cravatt et al., 1996b; 2001; Walker et al., 2002). All elements of this lipid signalling system are widely and densely expressed in the mammalian immune system and brain (Herkenham et al., 1990; Onaivi et al., 2006; Stella, 2009). As such, endocannabinoid regulation of immune function represents an important therapeutic target for a number of peripheral and central inflammatory disorders (Di Marzo et al., 2004; Baker et al., 2007; Centonze et al., 2007; Finn, 2009; Orgado et al., 2009).

Enhancing endocannabinoid tone has been proposed as an alternative means of activating cannabinoid receptors without concomitant overt psychotropic effects associated with potent synthetic cannabinoid receptor agonists. Enhancing endocannabinoid tone via FAAH or MAGL inhibition elicits anti-inflammatory effects in several animal models (Holt et al., 2005; Jayamanne et al., 2006; Comelli et al., 2007; Alhouayek et al., 2011; Booker et al., 2011). *In vitro* studies suggest that endocannabinoids elicit anti-inflammatory effects comparable to those of exogenous cannabinoids. Increasing AEA tone, either directly or via inhibition of its degradation or uptake, has been demonstrated to reduce the levels of pro-inflammatory cytokines and inflammatory mediators such as TNFα, IL-1β and nitric oxide, and enhance the release of the anti-inflammatory cytokine IL-10 *in vitro* (Puffenbarger et al.,

2000; Chang et al., 2001; Facchinetti et al., 2003; Ortega-Gutierrez et al., 2005; Tham et al., 2007; Correa et al., 2009; Correa et al., 2010). However, AEA has also been demonstrated to enhance IL-6 in astrocyte cultures (Molina-Holgado et al., 1998; Ortega-Gutierrez et al., 2005). Previous studies from our group have reported that the endocannabinoid re-uptake inhibitor AM404, attenuates LPS-induced increases in plasma IL-1β and IL-6 levels while concurrently augmenting TNFα levels (Roche et al., 2008). Comparably, De Laurentiis and co-workers recently demonstrated that AEA activation of hypothalamic CB_1 receptors facilitates LPS-induced increases in plasma TNFα levels (De Laurentiis et al., 2010). However, the effects of endocannabinoids on neuroinflammatory responses *in vivo* have not been examined in detail. Panikashvili and colleagues showed that 2-AG activation of CB_1 receptors inhibits TNFα, IL-1β and IL-6 mRNA in the brain and protects against closed head injury (Panikashvili et al., 2005; 2006). Enhanced AEA levels following inhibition of endocannabinoid re-uptake attenuated pro-inflammatory responses in the spinal cord and ameliorated motor symptoms in an animal model of multiple sclerosis (Mestre et al., 2005). The present study investigated the effect of the selective FAAH inhibitor URB597 (Kathuria et al., 2003; Fegley et al., 2005; Piomelli et al., 2006), on LPS-induced changes in cytokine expression in the hypothalamus, an important site of cytokine-mediated regulation of physiological function and stress responses, and compared those to effects observed in the periphery (plasma and spleen).

The mechanisms by which endocannabinoids mediate their neuroimmunomodulatory effects remain largely unknown. The neuroprotective and anti-inflammatory effect of 2-AG following closed head injury has been reported to be associated with a CB_1 receptor-mediated decrease in NFκB activation (Panikashvili et al., 2005). *In vitro* studies have suggested that the anti-inflammatory effects of AEA may be mediated by inhibition of NFκB activation and

enhanced production of the anti-inflammatory cytokine IL-10 (Correa et al., 2010). Cannabinoid ligands have also been reported to enhance suppressor of cytokine signalling (SOCS) 1 and SOCS3 gene expression in the periphery (Lavon et al., 2003; Caraceni et al., 2009) and inhibit SOCS3 activation in microglial cell culture (Kozela et al., 2010). However, no studies to-date have reported on the effects of endocannabinoids on SOCS signalling in the brain. In addition, it is well known that glucocorticoids are potent inhibitors of inflammatory responses and recent studies have demonstrated that the endocannabinoid system is an important regulator of stress-related neuroendocrine activity (for review see (Cota, 2008; Steiner and Wotjak, 2008; Gorzalka and Hill, 2009). Thus, a further aim of this study was to examine if alterations in LPS-induced cytokine expression in the hypothalamus following the inhibition of FAAH were associated with changes in NFκB or SOCS3 expression in the hypothalamus or alterations in plasma corticosterone levels.

EXPERIMENTAL PROCEEDURES

Animals

Experiments were carried out on male Sprague Dawley rats (weight 220-260g; Harlan, UK), housed singly in plastic bottomed cages (45 X 25 X 20 cm) containing wood shavings as bedding. The animals were maintained at a constant temperature (21 ± 2°C) under standard lighting conditions (12:12 h light–dark, lights on from 0800 to 2000 h). All experiments were carried out during the light phase between 0830 h and 1500 h. Food and water were available *ad libitum*. Animals were habituated to handling and received an intraperitoneal (i.p.) injection of sterile saline (0.89% NaCl) for 3-4 days prior to experimentation in order to minimise the influence of the injection procedure on biological endpoints. The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland, Galway under licence from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609.

Experimental design

Rats were randomly assigned to one of 4 treatment groups: Vehicle + Saline (n = 5); Vehicle + LPS (n = 8); URB597 + Saline (n = 7); URB597 + LPS (n = 8). Rats were injected with URB597 (1 mg/kg i.p. Cayman Chemicals, Estonia) or vehicle (ethanol: cremophor: saline; 1:1:18) in an injection volume of 2 ml/kg. The dose of URB597 was chosen based on its effectiveness at attenuating stress-induced activation of the HPA axis and nociceptive behaviour (Patel et al., 2004; Holt et al., 2005). The time of URB597 administration was determined based on the finding that inhibition of FAAH peaks 1 hour post i.p. injection and decreases thereafter (Kathuria et al., 2003; Fegley et al., 2005). Lipopolysaccharide (LPS: 100 μg/kg (Sigma B0111:B4)) or saline vehicle (sterile 0.89% NaCl) was administered at a

volume of 1 ml/kg, 30 minutes following URB597/vehicle. The dose and time of LPS administration were chosen on the basis of previous work within our laboratory demonstrating enhanced cytokine levels in the periphery and brain (Roche et al., 2006; 2008). Blood samples were taken 2 hours post LPS or saline administration, via cardiac puncture into a heparinized syringe under CO_2 anaesthesia. Blood samples were centrifuged at 14,000g for 15 min at 4°C to obtain plasma which was removed and stored at -80°C until cytokine and corticosterone determination. In addition, spleen and hypothalamus were excised, dissected in half, weighed and snap-frozen and stored at -80^{o}C until assayed for endocannabinoids, N-acylethanolamines and cytokines.

Quantitation of endocannabinoids and entourage N-acylethanolamines in hypothalamic and spleen tissue using liquid chromatography - tandem mass spectrometry (LC-MS/MS)

Quantitation of endocannabinoids and N-acylethanolamine was essentially as described previously (Olango et al., 2011). In brief, each hypothalamic or spleen sample was first homogenised in 400µL 100% acetonitrile containing known fixed amounts of deuterated internal standards (0.014 nmol AEA-d8, 0.48nmol 2-AG-d8, 0.016nmol PEA-d4, 0.015nmol OEA-d2). Homogenates were centrifuged at 14,000g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness. Lyophilised samples were re-suspended in 40µl 65% acetonitrile and 2µl were injected onto a Zorbax® C18 column (150 × 0.5mm internal diameter) from a cooled autosampler maintained at 4°C (Agilent Technologies Ltd, Ireland). Mobile phases consisted of A (HPLC grade water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12µl/min. Reversed-phase gradient elution began initially at 65% B and over 10min was ramped linearly up to 100% B. At 10min, the gradient was held at 100% B up to 20min. At 20.1min,

the gradient returned to initial conditions for a further 10mins to re-equilibrate the column. The total run time was 30min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.4min, 12.9min, 14.4min and 15.0min respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Instrument conditions and source parameters including fragmentor voltage and collision energy were optimised for each analyte of interest prior to assay of samples. Quantitation of target endocannabinoids was achieved by positive ion electrospray ionization and multiple reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H+] of the analytes of interest and the deuterated forms of the internal standards. Precursor and product ion mass-to-charge (m/z) ratios for all analytes and their corresponding deuterated forms were as follows: PEA (m/z = 300.3-62.1); PEA-d4 (m/z = 304.3-62.1); OEA (m/z = 326.3-62.1); OEA-d2 (m/z = 328.3-62.1); AEA (m/z = 348.3-62.1); AEA-d8 (m/z = 356.3-63.1); 2-AG (m/z = 379.3-287.2); 2-AG-d8 (m/z = 387.3-294.2). Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd, Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng per mg of tissue by dividing by the weight of the tissue. To express values as nmol or pmols per mg the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmole or pg/pmole. Linearity (regression analysis determined R^2 values of 0.99 or greater

for each analyte) was determined over a range of 18.75ng to 71.5fg except for 2-AG which was 187.5ng-715fg. The limit of quantification was 1.32pmol/g, 12.1pmol/g, 1.5pmol/g, 1.41pmol/g for AEA, 2-AG, PEA and OEA respectively.

Analysis of inflammatory mediators using real-time PCR

RNA was extracted from hypothalamic tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany). Genomic DNA contamination was removed with the addition of DNase to the samples according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used to quantify the gene of interest and real-time PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, UK), as previously described (O'Sullivan et al., 2009; McNamee et al., 2010). Assay IDs for the genes examined were as follows: IL-1β (Rn00580432_m1), TNF-α (Rn99999017_m1), IL-6 (Rn00561420_m1), IL-10 (Rn00563409_m1), IκBα (Rn01473658_g1) and SOCS3 (Rn00585674_s1). PCR was performed using Taqman Universal PCR Master Mix and samples were run in duplicate. The cycling conditions were 90^{o}C for 10 min and 40 cycles of 90^{o}C for 15 min followed by 60^{o}C for 1 min. β-actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the ΔΔCT method.

Determination of cytokine protein levels

Plasma and spleen TNFα, IL-1β, IL-6 and IL-10 concentrations were determined using specific rat enzyme-linked immunosorbent assays (ELISAs) performed using antibodies and standards obtained from R & D Systems, UK as previously described (Roche et al., 2006;

2008). Briefly, maxisorb microtitre plates were coated with goat or mouse anti-rat cytokine antibodies (0.8-4 μg/ml in phosphate-buffered saline [PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM; pH 7.4]) for 20 hours at 22°C. Plates were then washed three times with wash buffer (0.05% Tween 20 in PBS, pH 7.4) and blocked at room temperature for an hour using reagent diluent (1% BSA in PBS, pH 7.4). Following three washes, 100 μl aliquots of samples or standards (0–5000 pg/ml) were added and plates were incubated at 22°C for 2 hours. After three washes, 100 μl of specific biotinylated anti-goat or mouse antibody (1:1000) was added to each well and incubated for 1 hour at 22°C. After three washes, 100 μl horseradish peroxidase conjugated to streptavidin (1:200) was added to each well and plates were incubated at 22°C for 20 minutes. Following three washes, 100 μl of tetramethylbenzidine substrate solution was added per well and after for 20 min, 50 μl of 1 M H_2SO_4 was added per well to stop the reaction and to facilitate colour development. Absorbance was read immediately at 450 nm. Bradford protein assay was used to determine protein concentration in spleen samples. Cytokine levels in plasma are expressed as pg/ml or in the spleen as pg/mg protein.

Corticosterone determination

Plasma corticosterone was assessed as previously described (Roche et al., 2006) using a Corticosterone EIA kit (Cayman Chemicals, Tallin, Estonia) and carried out in accordance with manufacturer's instructions. The limit of detection of the assay was 30pg/ml and data were expressed as ng/ml plasma.

Statistical Analysis

SPSS statistical package was used to analyse all data. Data were analysed using two-way analysis of variance (ANOVA) with the factors of URB597/vehicle and LPS/Saline. *Post-*

hoc analysis was performed using Fisher's LSD test when appropriate. Data were considered significant when $P<0.05$. Results are expressed as group means + standard error of the mean (SEM).

RESULTS

Enhanced AEA, OEA and PEA in the hypothalamus following systemic administration of URB597 is accompanied by alterations in the expression of inflammatory mediators

Levels of AEA [$F_{1,22}$ = 44.51 P <0.001], OEA [$F_{1,22}$ = 97.74 P <0.001] and PEA [$F_{1,22}$ = 62.36 P <0.001], but not 2-AG, were significantly increased in the hypothalamus following systemic administration of URB597 (Figure 1). LPS did not significantly alter the concentration of the endocannabinoids or the related N-acylethanolamine compounds, nor did it alter the URB597-induced increase in the levels of AEA, OEA or PEA.

LPS significantly enhanced cytokine (IL-1β: $F_{1,22}$ = 54.62 P <0.001 , TNFα: $F_{1,23}$ = 29.48 P <0.001; IL-6: $F_{1,22}$ = 19.63 P <0.001 and IL-10: $F_{1,22}$ = 30.20 P <0.001), IκBα [$F_{1,20}$ = 349.38 P <0.001] and SOCS3 [$F_{1,21}$ = 60.12 P <0.001] mRNA expression in the hypothalamus (Figure 2). URB597 did not significantly alter the expression of immune mediators in the absence of LPS. However, URB597 did attenuate the LPS-induced increase in IL-1β expression [interaction effect: $F_{1,22}$ = 6.86 P = 0.016] and concurrently augmented LPS-induced SOCS-3 expression [interaction: $F_{1,20}$ = 8.30 P = 0.009]. Although URB597 enhanced and reduced the LPS-induced increase in IL-6 and IL-10 expression respectively, ANOVA revealed that these effects failed to reach statistical significance. There was no significant effect of URB597 on LPS-induced expression in TNFα or IκBα mRNA in the hypothalamus.

Inhibition of FAAH by URB597 increases levels of AEA, OEA and PEA in the spleen without altering LPS-induced increases in cytokine levels

Similar to effects observed in the hypothalamus, URB597 increased AEA [$F_{1,22}$ = 44.51 P <0.001], OEA [$F_{1,22}$ = 97.74 P <0.001] and PEA [$F_{1,22}$ = 62.36 P <0.001], but not 2-AG, levels in the spleen, an effect not altered in the presence of LPS (Figure 3A-D). LPS significantly increased the levels of the pro-inflammatory cytokines, IL-1β [$F_{1,17}$ = 264.23 P <0.001], TNFα [$F_{1,21}$ = 30.79 P <0.001] and IL-6 [$F_{1,18}$ = 138.39 P <0.001] and the anti-inflammatory cytokine IL-10 [$F_{1,23}$ = 13.43 P = 0.001] in the spleen (Figure 3E-H). URB597 did not alter the levels of pro- or anti-inflammatory cytokines in the spleen in the presence or absence of LPS.

URB597 does not significantly alter LPS-induced increases in pro-inflammatory cytokines or corticosterone levels in the plasma

LPS induced a significant increase in the levels of pro-inflammatory cytokine levels [IL-1β: $F_{1,17}$ = 158.15 P <0.001; TNFα: $F_{1,18}$ = 10.10 P =0.005 and IL-6: $F_{1,18}$ = 97.52 P <0.001] and corticosterone [$F_{1,18}$ = 171.95 P <0.001] in the plasma (Figure 4 and 5). URB597 did not significantly alter plasma pro-inflammatory cytokine levels, in the presence or absence of LPS. LPS-induced corticosterone levels were slightly lower in URB597-treated animals than in vehicle-treated rats, however this effect was not statistically significant.

DISCUSSION

The present study demonstrated that systemic administration of the FAAH inhibitor URB597 increased AEA, OEA and PEA levels centrally, in the hypothalamus, and peripherally, in the spleen. URB597 attenuated the LPS-induced increase in IL-1β expression while concurrently enhancing LPS-induced SOCS3 expression in the hypothalamus. There was no effect of FAAH inhibition on cytokine levels in the periphery (plasma or spleen) or on plasma corticosterone levels. The current study demonstrates an important role for FAAH in the modulation of central neuroimmune processes associated with acute inflammation.

Correlating with earlier studies (Felder et al., 1996), basal levels of the fatty acid amides, AEA, OEA and PEA were lower in the spleen than in the brain. Despite this, systemic administration of the FAAH inhibitor URB597, reliably and robustly increased levels of AEA and the related N-acetyletholamines, OEA and PEA, in both the spleen and hypothalamus. These findings corroborate previous studies demonstrating an increase in these fatty acid amides in whole brain extracts and liver following administration of URB597 (Kathuria et al., 2003; Fegley et al., 2005). FAAH is also known to metabolise 2-AG (Di Marzo et al., 1998), although not to the same extent as MAGL (Dinh et al., 2002), however URB597 did not alter 2-AG levels in either the hypothalamus or spleen. This confirms that the dose of URB597 used in the present study selectively enhanced fatty acid amide levels. URB597-induced increases in the fatty acid amides were not altered in the presence of LPS. This may be somewhat surprising in light of *in vitro* evidence indicating that LPS increases AEA and 2-AG levels, an effect mediated by inhibition of FAAH and MAGL activity and/or enhancement of AEA biosynthesis (Varga et al., 1998; Di Marzo et al., 1999; Maccarrone et al., 2001; Liu et al., 2003). *In vivo,* LPS has been demonstrated to increase AEA synthesis

(Fernandez-Solari et al., 2006) and reduce 2-AG levels (Borges et al., 2011) in the rat hypothalamus. Although the dose of LPS (100µg/kg) and time post injection (2hrs) used in both the present and former (Borges et al., 2011) studies are identical, methodological differences such as strain of rat (Sprague Dawley vs Wistar), strain of LPS (0111:B4 vs 026:B6) or time of day of the injections (light phase vs just prior to dark phase) may account for the discrepancy between studies. In addition, in the study demonstrating that LPS increased AEA synthesis (Fernandez-Solari et al., 2006), LPS was administered at a dose 50 times greater than that given in the present study. To our knowledge, the effects of LPS on levels of OEA or PEA have not been reported, however, should LPS inhibit FAAH activity, it would have been expected that all three fatty acid amides would be enhanced. The results of this study argue against an LPS-induced increase in endocannabinoid synthesis or FAAH or MAGL inhibition at the dose used in the current study.

It is well established that endotoxin administration is associated with the induction of inflammatory cytokines in the brain and periphery (Breder et al., 1994; Pitossi et al., 1997; Konsman et al., 1999; Turrin et al., 2001; Roche et al., 2006; 2008), findings confirmed in the present study. Increased circulating pro-inflammatory cytokines communicate with the brain via many routes (diffusion into brain across the blood brain barrier deficient areas, sensory signals and vagus nerve stimulation) and induce cytokine synthesis within the CNS, which leads to a state of acute neuroinflammation and sickness behaviour. Thus, modulation of peripheral cytokines can impact on inflammatory signals sent to the brain. Although URB597 enhanced the levels of AEA, OEA and PEA in both the spleen and hypothalamus, it failed to alter cytokine levels in the periphery. URB597, administered both systemically and i.c.v., has previously been shown to augment LPS-induced increases in TNFα plasma levels (Roche et al., 2008; De Laurentiis et al., 2010). LPS-induced TNFα release in the plasma is

primarily controlled by the CNS (Mastronardi et al., 2001) and recent evidence indicates that AEA induced augmentation of LPS-stimulated increases in TNFα is mediated by hypothalamic CB_1 receptors (De Laurentiis et al., 2010). However, a higher dose of URB597 (1mg/kg) was used in the current study in comparison to our earlier study (0.6mg/kg) (Roche et al., 2008) which may have resulted in increased fatty acid amide levels, in particular OEA and PEA. Indeed, it is possible that the effects of OEA and/or PEA may have counteracted the effects of AEA on LPS-induced TNFα levels in the periphery. For example, PEA is known to elicit potent anti-inflammatory effects, including inhibition of LPS-induced increases in plasma TNFα levels (Berdyshev et al., 1998; Hoareau et al., 2009). In any case, the present results indicate that the effects of URB597 on LPS-induced cytokine expression in the hypothalamus appear to be mediated at the level of the CNS rather than secondary to effects on peripheral cytokines.

Cytokines within the hypothalamus have been shown to be responsible for many aspects of the sickness response following endotoxin administration (for reviews see (Beishuizen and Thijs, 2003; Conti et al., 2004; Dantzer, 2009)). For example, IL-1β has been shown to mediate fever (Murakami et al., 1990) and hypophagia (Kent et al., 1994) in response to LPS, effects which can be attenuated by AEA (Hollis et al., 2011). The present study demonstrates that enhanced levels of AEA, OEA and PEA in the hypothalamus following URB597 were associated with an attenuation of LPS-induced IL-1β, but not TNFα or IL-6 expression. Thus, AEA-induced inhibition of IL-1β expression may prevent/attenuate the fever and hypophagia associated with acute inflammation. Pro-inflammatory cytokines such as IL-1β and TNFα are also responsible for activation of the HPA axis and consequently glucocorticoid release following LPS (Beishuizen and Thijs, 2003). In addition, endocannabinoid modulation of neuroendocrine activity has been the topic of several studies

(for reviews see (Cota, 2008; Steiner and Wotjak, 2008; Gorzalka and Hill, 2009)) with increasing evidence that endocannabinoids act to inhibit stress-induced HPA axis activation (Patel et al., 2004; Di et al., 2005; Evanson et al., 2010). The present study demonstrated that enhanced AEA tone following URB597 failed to alter LPS-induced increases in plasma corticosterone levels or the expression of TNFα in the hypothalamus. Therefore, hypothalamic TNFα may underlie the LPS-induced increase in plasma corticosterone, an effect not altered by URB597. **However, it is also possible that URB597 may have elicited an effect on HPA activation and corticosterone levels at an earlier time point than that examined in the present study. It has been previously shown that URB597 (0.1-1mg/kg) attenuates restraint-stress induced increase in corticosterone levels 1 hr post administration (Patel et al., 2004) and that blockade of the CB_1 receptor enhances stress-induced increases in corticosterone up to 90 minutes post stress (Patel et al., 2004; Hill et al., 2011). As such, by examining corticosterone levels 2hrs post LPS, the effects of cytokine modulation by URB597 on HPA activation may have been missed**. Enhancing anti-inflammatory cytokine levels such as IL-10 (systemically and centrally) has also been shown to attenuate LPS-induced hypophagia, fever, reduced motor activity and energy expenditure (Ledeboer et al., 2002; Hollis et al., 2010). The present study demonstrated that URB597 reduced LPS-induced expression of IL-10, in the hypothalamus. These findings are in contrast with *in vitro* data showing that AEA increases LPS/IFNγ-induced IL-10 levels in microglia (Correa et al., 2010). It is possible that the effects of URB597 on IL-10 observed in the present study may be mediated by OEA and/or PEA and not AEA. In addition, the present findings suggest that IL-10 is not responsible for the reduction in LPS-induced IL-1β expression observed following URB597 administration.

In an effort to examine possible molecular mechanisms by which enhanced endocannabinoid tone may modulate cytokine expression within the hypothalamus, this study also investigated the effect of URB597 on the expression of IκBα, an indirect measure of NFκB signalling (Read et al., 1994), and SOCS3. Although AEA has previously been demonstrated to modulate NFκB activation (Sancho et al., 2003; Nakajima et al., 2006; Correa et al., 2010), LPS-induced IκBα expression was not altered by URB597 in the present study. Therefore it is unlikely that NFκB signalling system is involved in the attenuation of cytokine expression in the hypothalamus following URB597 administration. In comparison, the present study demonstrated that FAAH inhibition results in an augmentation of LPS-induced SOCS3 expression. SOCS proteins represent a rapid self-regulating mechanism to modulate cytokine signalling. Previous studies have demonstrated that cannabinoid ligands enhance SOCS3 expression in the periphery (Lavon et al., 2003; Caraceni et al., 2009) and inhibit SOCS3 activation in cultured microglial cells (Kozela et al., 2010). However, this is the first study to demonstrate endocannabinoid-induced changes in SOCS3 expression following an immune challenge *in vivo*. The expression of SOCS3 is primarily regulated by activation of signal transducer and activation of transcription (STAT)-3 by IL-6 GP130 and IL-10 cytokines, although other signalling cascades such as NFκB and MAPK are also known to be involved (Qin et al., 2007; Baker et al., 2009). Our data demonstrated that LPS-induced IL-6 expression in the hypothalamus was enhanced, although not significantly, following URB597. Thus the augmentation of LPS-induced SOCS3 expression may be the result of enhanced IL-6 signalling following the inhibition of FAAH. The primary function of SOCS3 is to inhibit signalling by IL-6 via inhibition of the JAK/STAT3 pathway, thus enhanced SOCS3 expression following URB597 may limit the pro-inflammatory action of this cytokine. In addition, IL-1β-induced transcription and activation of NFκB and the MAPKs is inhibited by SOCS3 (Karlsen et al., 2004; Frobose et al., 2006). SOCS3 also mediates some

of the anti-inflammatory effects of IL-10 (Berlato et al., 2002; Qin et al., 2007), however as URB597 attenuated rather than enhanced LPS-induced IL-10, it seems unlikely that the enhanced SOCS3 expression in the present study was induced following IL-10 signalling. It should also be noted that LPS induces increases in other cytokine-like molecules such as leptin which are also capable of inducing STAT-3 phosphorlyation and SOCS3 expression in the hypothalamus (Hubschle et al., 2001), an effect proposed to underlie, at least in part, LPS-induced hypophagia (Borges et al., 2011). Further studies are required to assess the contribution of the individual fatty acid amides to the activation of SOCS3 and the downstream consequences of this activation.

In summary, the present study demonstrates an important role for FAAH in the modulation of neuroinflammatory responses. As the hypothalamus is a critical site in the regulation of anorexia, fever, neuroendocrine and sympathetic activity in response to an acute inflammatory stress, the present findings may have important implications in targeting the endocannabinoid system for the treatment of stress-related neuroinflammatory and psychiatric disorders.

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Figure Legends

Figure 1: Systemic administration of URB597 enhances the levels of (A) AEA, (C) OEA and (D) PEA, but not (B) 2-AG, in the hypothalamus. Data expressed as mean + SEM. (n = 4-8 per group). **P<0.01 vs. vehicle-saline. $^{++}$P<0.01 vs. vehicle-LPS..

Figure 2: URB597 modulates LPS-induced changes in inflammatory gene expression in the hypothalamus. URB597 attenuates LPS-induced increase in (A) IL-1β and augments the LPS-induced increase in (F) SOCS3 expression. Although URB597 appeared to modulate the LPS-induced increase in (C) IL-6 and (D) IL-10 expression, analysis revealed that this failed to reach statistical significance. There was no effect of URB597 on (B) TNF-α or (E) IκBα expression, in the presence or absence of LPS. Data expressed as mean + SEM. (n = 4-8 per group). mRNA data is expressed as fold-change vs. vehicle-saline. **P<0.01 *P<0.05 vs. vehicle-saline. $^{\#\#}$P<0.01 $^{\#}$P<0.05 vs. URB597-saline. $^{++}$P<0.01 vs. vehicle-LPS.

Figure 3: URB597 increases the concentration of (A) AEA, (C) OEA and (D) PEA, but not 2-AG in the spleen. LPS induced an increase in (E) IL-1β, (F) TNF-α and (G) IL-6 or (H) IL-10, an effect not altered by URB597. Data expressed as mean + SEM. (n = 4-8 per group). **P<0.01 vs. vehicle-saline. $^{\#\#}$P<0.01 vs. URB597-saline. $^{++}$P<0.01 vs. vehicle-LPS.

Figure 4: URB597 does not significantly alter LPS-induced cytokine levels in the plasma. LPS induced an increase in (A) IL-1β, (B) TNF-α and (C) IL-6. There was no significant effect of URB597 on cytokine levels in the presence or absence of LPS. Data expressed as mean + SEM. (n = 4-7 per group). **P<0.01 vs. vehicle-saline. $^{\#\#}$P<0.01 vs. URB597-saline.

Figure 5: URB597 does not significantly alter LPS-induced corticosterone levels in the plasma. Data expressed as mean + SEM. (n = 4-7 per group). **P<0.01 vs. vehicle-saline. ##P<0.01 vs. URB597-saline.

Figure1

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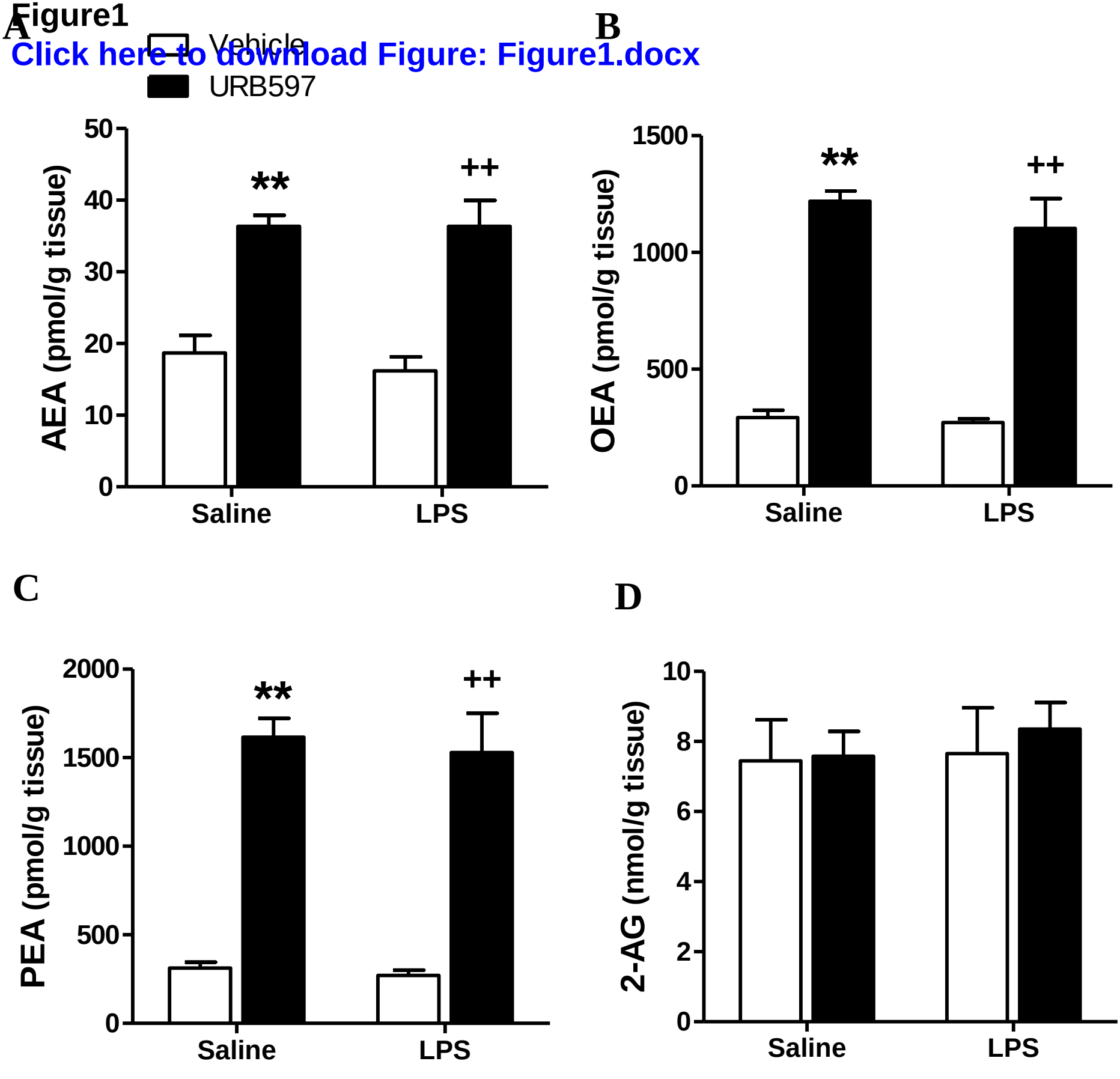

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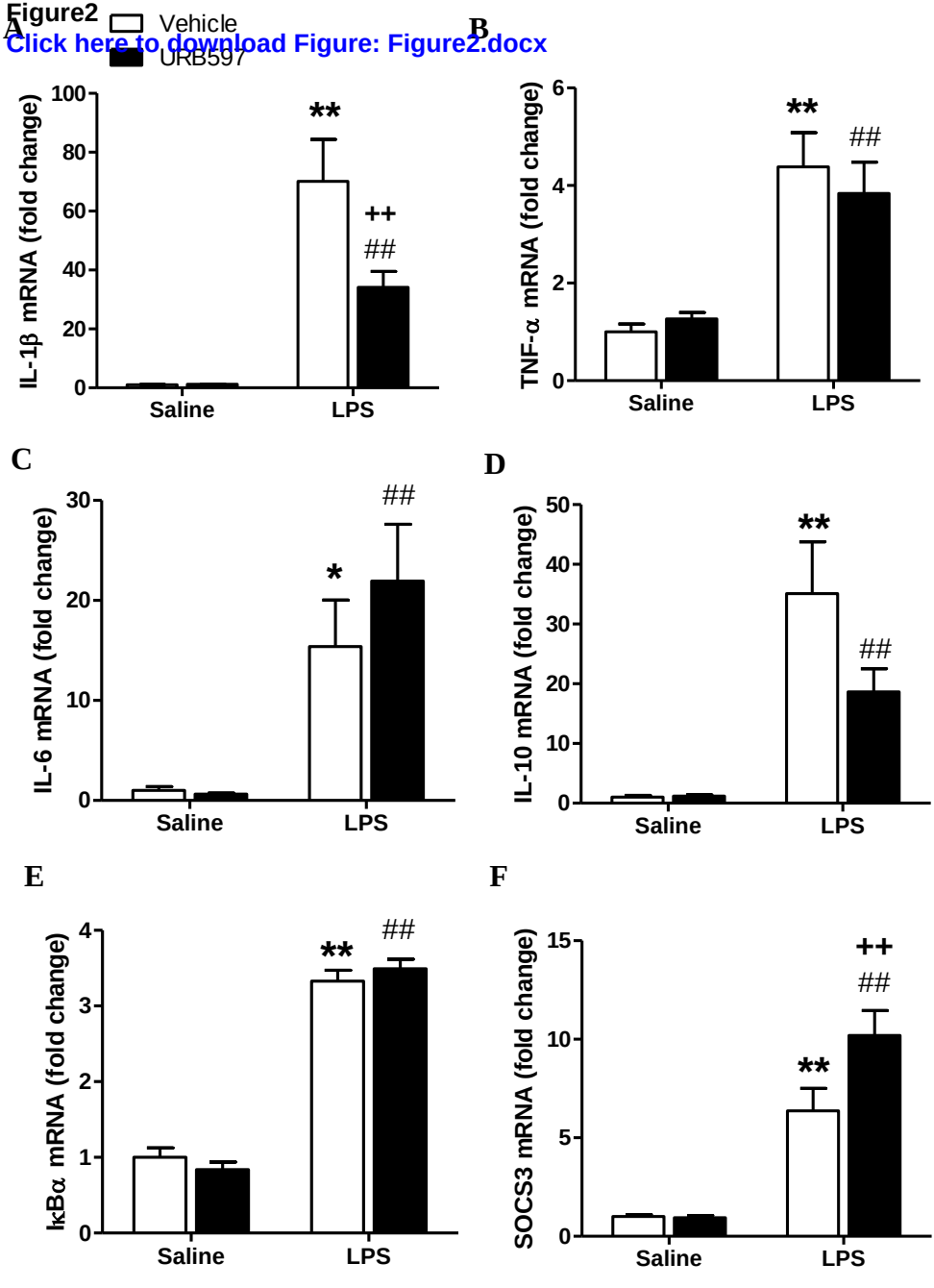

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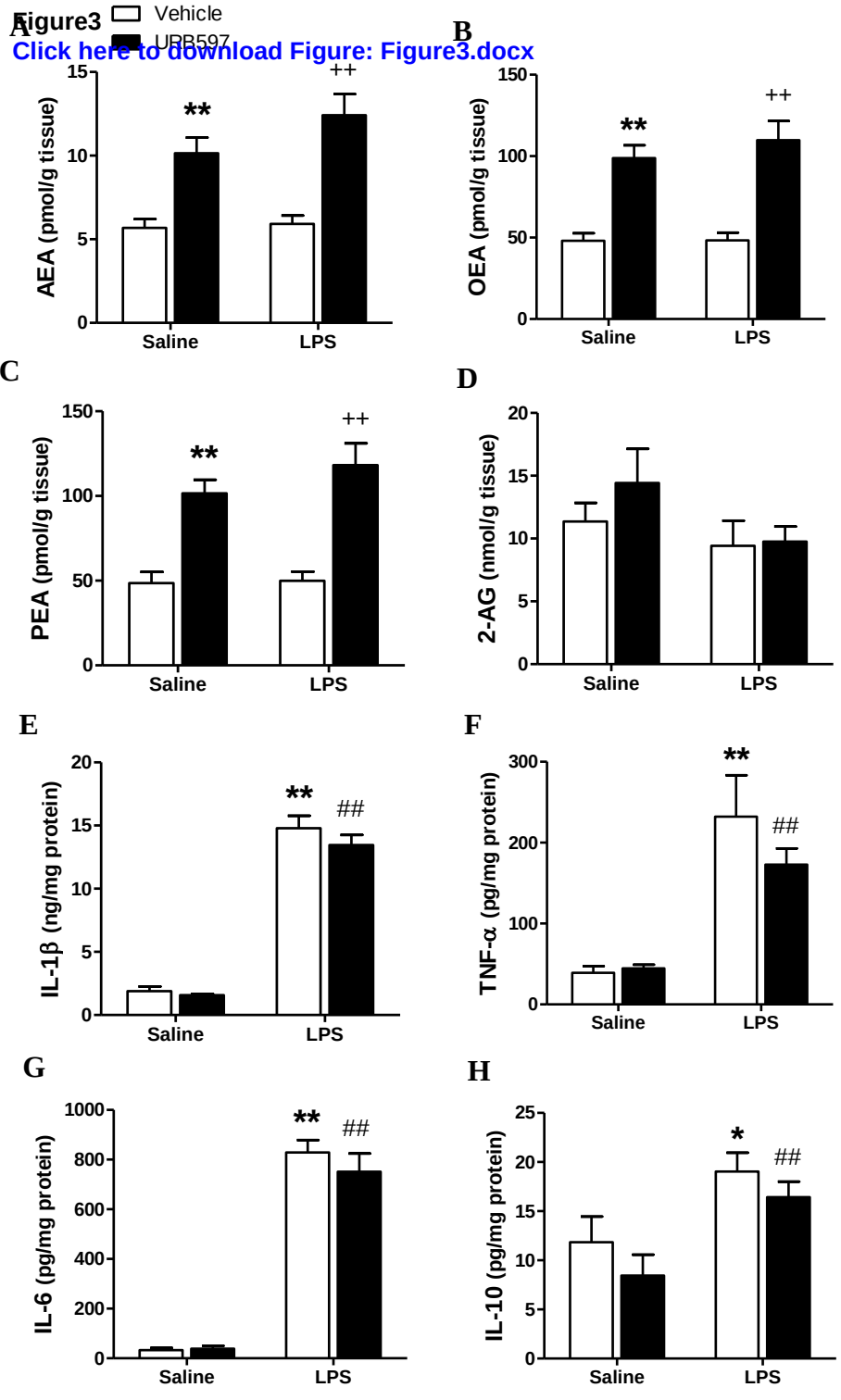

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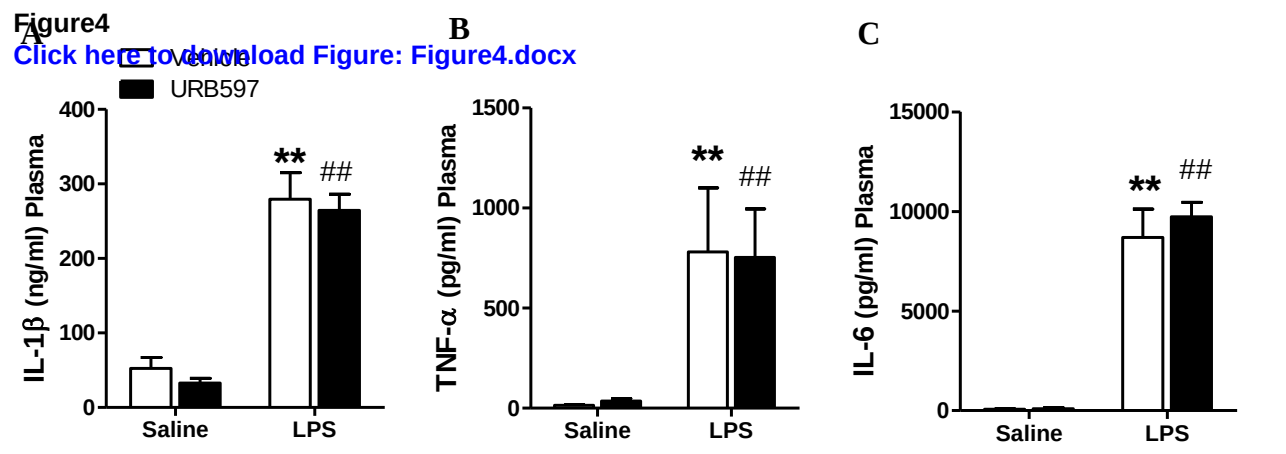

Figure5

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