In vitro and in vivo evidence for anti-inflammatory properties of 2-methoxyestradiol

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Abbreviations: 2MEO, 2-methoxyestradiol; BAL, bronchoalveolar lavage; DEX, dexamethasone; E2, 17β-estradiol; IFNγ, interferon-γ; IL-6, interleukin-6; LPS, lipopolysaccharide; PGE2, prostaglandin E2; TNF-α, tumour necrosis factor-α.

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Abstract

2-Methoxyestradiol (2MEO) is an endogenous metabolite of 17β-estradiol that interacts with estrogen receptors and microtubules. It has acute anti-inflammatory activity in animal models that is not attributable to known anti-proliferative or anti-angiogenic actions. As macrophages are central to the innate inflammatory response, we examined whether suppression of macrophage activation by 2MEO could account for some of its anti-inflammatory effects. Inflammatory mediator production stimulated by lipopolysaccharide (LPS) and interferon-γ in the J774 murine macrophage cell line or human monocytes was measured after treatment with 2MEO or the anti-inflammatory agent dexamethasone. The effect of these agents on LPS-induced acute lung inflammation in mice was also examined. 2MEO suppressed J774 macrophage interleukin-6 and prostaglandin E2 production (by 30% and 47% respectively at 10 µM) and human monocyte tumour necrosis factor-α production (by 60% at 3 µM). Estradiol had no effect on J774 macrophage activation, nor did the estrogen receptor antagonist ICI 182,780 prevent the effects of 2MEO. The actions of 2MEO were not mimicked by the microtubule interfering agents colchicine or paclitaxel. In mice exposed to LPS, bronchoalveolar lavage protein content, a measure of vascular leak and epithelial injury, was reduced to a comparable extent (~54%) by treatment with 2MEO (150 mg·kg⁻¹) or dexamethasone (1 mg·kg⁻¹). Additionally, 2MEO reduced LPS-induced interleukin-6 gene expression. Thus, 2MEO modulates macrophage activation in vitro and has high-dose acute anti-inflammatory activity in vivo. These findings are consistent with the acute anti-inflammatory actions of 2MEO being mediated in part by suppression of macrophage activation.
Introduction

2-Methoxyestradiol (2MEO), an endogenous metabolite of the sex steroid 17β-estradiol, is present in low concentrations (<0.2 nM) in the blood and urine (Berg et al., 1983). 2MEO attracted considerable interest as a potential anti-cancer therapeutic after it was found to inhibit the in vitro proliferation and migration of bovine brain capillary endothelial cells and the in vivo neovascularisation and growth of solid tumours (Fotsis et al., 1994). The anti-proliferative and anti-angiogenic actions of 2MEO have since been characterised in a variety of cell types and in vivo tumour models (reviewed in Sutherland et al., 2007). An oral formulation was well tolerated in Phase 1 and 2 clinical trials, and although the development of 2MEO as an anti-cancer agent stalled due to low potency and poor bioavailability (LaVallee et al., 2008), the agent remains in development for other indications, such as rheumatoid arthritis.

The mechanisms of the anti-proliferative and anti-angiogenic actions of 2MEO on tumour and endothelial cells have been investigated extensively (Mooberry, 2003). 2MEO disrupts microtubule function, inhibits HIF-1α transcription factor activity and cell cycle progression into S-phase, and activates a number of pro-apoptotic signalling pathways (Hughes et al., 2002; Sutherland et al., 2007). 2MEO is also a low affinity agonist for the estrogen receptor (Kd ~100 nM cf. 17β-estradiol Kd ~0.2 nM). However, the plasma levels of 2MEO required for anti-tumour activity (0.1–10 µM) have been shown to have a significant degree of estrogenic activity, both in vitro and in vivo (Sutherland et al., 2005). In addition to microtubules and estrogen receptors, a number of novel 2MEO binding proteins have been detected by affinity chromatography, but the functional significance of these is yet to be determined (Ho et al., 2006).
In addition to anti-tumour actions, 2MEO displays anti-inflammatory activity in animal models of rheumatoid arthritis (Josefsson and Tarkowski, 1997; Issekutz and Sapru, 2008; Plum et al., 2009), chronic airway inflammation (Huerta-Yepez et al., 2008) and pulmonary fibrosis (Langenbach et al., 2007; Tofovic et al., 2009). In many of these chronic models, the effects of 2MEO are attributed to anti-angiogenic activity. However, 2MEO also displays anti-inflammatory activity that may be independent of its effects on blood vessel development. Issekutz and Sapru (2008) and Plum et al. (2009) report inhibition of synovial leukocyte infiltration in the absence of changes in vascularity. Plum et al. also observed inhibition of gene expression for inflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α). Furthermore, in estrogen-deficient rats, 2MEO reduced the bleomycin-induced influx of macrophages into the lungs (Tofovic et al., 2009). These findings raise the possibility that 2MEO might directly regulate cells involved in the inflammatory response.

Monocytes and macrophages are essential for innate and adaptive host defence and play a central role in the inflammatory response. Activation by stimuli such as lipopolysaccharide (LPS) and interferon-γ (IFNγ) results in the production of inflammatory signalling mediators that include nitric oxide, a free radical with antimicrobial activity, generated by inducible nitric oxide synthase (NOS-2); prostaglandin E2 (PGE2), a locally-acting vasodilator generated by cyclo-oxygenase (COX)-2 and PGE isomerase; and pluripotent cytokines such as IL-6 and TNF-α that are involved in the acute phase systemic inflammatory response. Inappropriate macrophage activation contributes to numerous pathological processes, including asthma, atherosclerosis, rheumatoid arthritis and septic shock. Thus, agents that regulate macrophage function may provide relief from these conditions.
Numerous studies have examined the actions of 17β-estradiol on macrophages. Such studies confirm a mechanistic role for high-affinity estrogen receptors, but yield conflicting reports as to the effects of 17β-estradiol on cell function, ranging from inhibition to promotion of activation (compare for example findings reported by Hayashi et al., 1998; Lu et al., 2004; Vegeto et al., 2004; Sakazaki et al., 2005). On the other hand, little attention has been given to the effects of 17β-estradiol metabolites, such as 2MEO, on macrophage function. 2MEO inhibits prostaglandin production by guinea-pig peritoneal macrophages (Stewart, 1999), and is cytotoxic to RAW 264.7 macrophage-derived osteoclasts (Maran et al., 2006). Coupled with the unexplained anti-inflammatory effects of 2MEO in animal models, we believe these findings warrant further investigation of the direct effects of 2MEO on macrophages.

The aims of this study were to characterise the effects of 2MEO on macrophage function in vitro, and to investigate whether the in vitro activity of 2MEO would translate to anti-inflammatory activity in vivo, in an acute model in which macrophages play a key role. We show that 2MEO suppresses LPS/IFNγ-induced activation of the J774 murine macrophage cell line and human monocytes, and suppresses acute LPS-induced airway inflammation in mice.
Methods

Mouse macrophage cell line culture and treatments

The J774 A.1 murine macrophage cell line (from American Type Culture Collection, Manassas, VA, USA) was cultured in phenol red-free Dulbecco’s Modified Eagle’s Medium supplemented with 5% v/v heat-inactivated foetal calf serum, 15 mM HEPES, 2 mM L-glutamine, 0.2% sodium bicarbonate, 50 IU·ml⁻¹ penicillin and 50 µg·ml⁻¹ streptomycin, maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were passaged by scraping. Cells were seeded on 6-well plastic culture plates at a density of 4.0 × 10⁴ cells·cm⁻² (for western blot analysis), on 24-well plates at 7.5 × 10⁴ cells·cm⁻² (for measurement of IL-6, TNF-α, PGE₂ and nitrite), and on 96-well plates at 9.0 × 10⁴ cells·cm⁻² (for crystal violet cell number assays). Although the cell-to-surface area ratio of cells in 6-well plates was considerably less than 24- or 96-well plates, it was found that cells became over-confluent if plated at higher densities. Cells were grown to 90–95% confluence over 48 h before the culture medium was replaced with fresh medium (2 ml/well on 6-well plates, 500 µl/well on 24-well plates, 200 µl/well on 96-well plates). Cells were then incubated in the fresh medium for a further 2 h prior to treatment. Before stimulation with LPS (1 µg·ml⁻¹) and IFNγ (0.1 IU·ml⁻¹), cells were pre-treated with 2MEO, 17β-estradiol, dexamethasone, colchicine, paclitaxel and/or ICI 182,780. Unstimulated control groups were included in all experiments. The final concentration of dimethylsulfoxide vehicle in the culture medium was controlled across all treatment groups at 0.10% v/v, a concentration previously found to have no effect on J774 cell function. At the end time point of each experiment (24 h after LPS/IFNγ stimulation, except for time course experiments), cell supernatants were collected and stored at −20°C for subsequent determination of IL-6, TNF-α, PGE₂ and nitrite concentrations.
**Human peripheral blood monocyte cell culture**

The use of human blood products was approved by the Human Ethics Committee of the University of Melbourne. Human monocytes were isolated from buffy coat packs of healthy donors, kindly supplied by the Australian Red Cross Blood Service. Following dextran sedimentation to minimise erythrocyte contamination (0.6% dextran T500 in saline for 1 h), buffy coat cell suspensions were washed twice in saline at $210 \times g$ before layering over a Lymphoprep gradient and centrifugation for 20 min at $470 \times g$ at room temperature without braking. The peripheral blood mononuclear cell layer was removed, resuspended to 50 ml in saline, washed three times at $210 \times g$, then resuspended in RPMI 1640 medium supplemented with 10% v/v heat-inactivated foetal calf serum, 15 mM HEPES, 2 mM l-glutamine, non-essential amino acid solution, 0.2% sodium bicarbonate, 1 mM sodium pyruvate, 50 IU·ml$^{-1}$ penicillin and 50 µg·ml$^{-1}$ streptomycin. Peripheral blood mononuclear cells were seeded on 24-well plastic culture plates at $2.0 \times 10^6$ cells·cm$^{-2}$ and allowed to adhere for 2–3 h at 37°C in a humidified atmosphere containing 5% CO$_2$, after which non-adherent cells were removed by four vigorous washings in pre-warmed saline. The remaining monocytes (average $0.5 \times 10^6$ cells·cm$^{-2}$) were incubated for 22 h prior to treatment as described above for J774 macrophages, with the exception that cells were stimulated with 100 ng·ml$^{-1}$ LPS and 10 ng·ml$^{-1}$ IFNγ, and that some cells were also treated with RU486.

**Animals**

All procedures conformed to the animal welfare guidelines of the National Health and Medical Research Council of Australia, and were approved by the Animal Ethics Committee of the University of Melbourne. Mice were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed at 20°C on a 12 h light-dark cycle with food (Purina mouse chow) and water available *ad libitum.*
Mouse model of LPS-induced acute lung injury

The mouse model of LPS-induced acute lung injury was performed as described previously (Szarka et al., 1997). Briefly, female BALB/c mice (9–11 weeks old; 20–25 g) in weight-matched treatment groups were lightly anaesthetised with methoxyflurane by inhalation, before intranasal instillation of 1 µg LPS in 35 µl saline vehicle. Saline/vehicle control mice received saline only. Two hours after LPS instillation, mice received an intraperitoneal injection of 2MEO, dexamethasone or vehicle (100 µl; 90% peanut oil and 10% dimethylsulfoxide). Twenty-four hours after LPS instillation, mice were killed with an intraperitoneal injection of sodium pentobarbital (150 mg·kg⁻¹) and the trachea was cannulated for bronchoalveolar lavage (BAL). Lungs were lavaged four times with 0.3 ml aliquots of saline. BAL fluid aliquots were pooled and stored on ice for cell counts and protein measurement. Following lavage, the lungs were removed, snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction. BAL cell counts were performed using a Neubauer haemocytometer, with ethidium bromide and acridine orange fluorescent stains used to determine cell viabilities. After cell counts had been performed, BAL fluid was centrifuged at 300 × g for 10 min and the supernatant collected and stored at −20°C until protein concentrations were measured using the Bio-Rad protein assay method, as described in the manufacturer’s instructions.

IL-6 and TNF-α ELISA

The concentrations of mouse and human IL-6 and TNF-α in macrophage and monocyte culture medium were measured using BD OptEIA ELISA sets or matched antibody pairs according to the manufacturers’ protocols. The limits of detection for mouse IL-6 and TNF-α
were 32 pg·mL⁻¹ and 16 pg·mL⁻¹ respectively. The limit of detection for both human IL-6 and TNF-α was 16 pg·mL⁻¹.

PGE₂ radioimmunoassay

The concentration of PGE₂ in the macrophage culture medium was measured by radioimmunoassay. Cell supernatant samples (diluted 1/4) and PGE₂ standards (0.02–40 ng·mL⁻¹) prepared in assay buffer (0.1% gelatin in 50mM Tris-HCl) were incubated overnight at 4°C with [³H]PGE₂ tracer (37 Bq ≈ 22 000 dpm/tube) and anti-PGE₂ (sufficient to give 30% maximal binding). The assay volume was 400 µl. Unbound [³H]PGE₂ was removed with 500 µl ice-cold dextran-coated charcoal (20 mg·mL⁻¹ charcoal in assay buffer containing 4 mg·mL⁻¹ heat-dissolved dextran) and subsequent centrifugation (10 min, 1880 ×g, 4°C). The supernatants, containing the bound [³H]PGE₂, were mixed with 4 ml liquid scintillation fluid and β-radiation emissions were measured in a scintillation counter (Packard 1600TR, PerkinElmer, Glen Waverley, VIC, Australia). PGE₂ concentrations were calculated in ng·mL⁻¹ by comparison of sample radioactivity (in disintegrations per minute) with that of the PGE₂ standards. The limit of detection for PGE₂ was 0.08 ng·mL⁻¹.

Nitrite assay

The concentration of nitrite in the macrophage culture medium was measured by adding 100 µl of Griess reagent (0.1% naphthylethylenediamine in dH₂O and 1% sulphanilamide in 5% v/v phosphoric acid, mixed 1:1 immediately before use) to 100 µl undiluted cell supernatant samples. The absorbance at 550 nm was measured using a microplate reader (Multiskan Ascent, Thermo Fisher Scientific, Milford, MA, USA). Nitrite concentrations were calculated in µM by comparison of sample absorbances with those of a series of sodium nitrite standard solutions (0–300 µM).
Western blot analysis of protein expression

Cell lysates for western blotting were prepared by washing cells twice with ice-cold phosphate-buffered saline followed by cell lysis in 700 µl of lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 7.5; 2 mM EDTA; 0.5% w/v deoxycholate; 1% v/v Triton X-100; 1% v/v protease inhibitor cocktail and 1% v/v phosphatase inhibitor cocktail) for 15 min on ice. Cell lysates were centrifuged for 10 min at 7800 \( \times g \) at 4°C, and the supernatants were collected and stored at –20°C until western blotting. Lysate protein concentrations were measured using the Bio-Rad protein assay method, as described in the manufacturer’s instructions. Cell lysate volumes corresponding to equivalent protein concentrations were mixed with reducing sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% v/v glycerol; 2% w/v sodium dodecyl sulphate; 0.0025% bromophenol blue; 1% v/v 2-mercaptoethanol) and boiled for 5 min prior to being subjected to electrophoresis on a 10% acrylamide gel. The resolved proteins were electroblotted onto Hybond nitrocellulose membranes by the Bio-Rad semi-dry transfer method, according to the manufacturer’s instructions. Membranes were stained with Ponceau Red to verify uniform protein transfer, then blocked with 5% skim milk in Tris-buffered saline + 0.1% Tween-20 (TBS-Tween; 10 mM Tris, pH 7.4; 75 mM NaCl; 0.1% Tween-20) for 1 h. Blocked membranes were incubated overnight at 4°C with COX-2 rabbit polyclonal antibody (diluted 1/2500), NOS-2 rabbit polyclonal antibody (diluted 1/1000), or \( \beta \)-actin mouse monoclonal antibody (diluted 1/10,000). Blots were washed three times in TBS-Tween prior to incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (sheep anti-rabbit IgG diluted 1/2500, or sheep anti-mouse IgG diluted 1/5000) for 1 h at room temperature. After three washes with TBS-Tween, the immunoreactive proteins were visualised by enhanced chemiluminescence (ECL reagent) prior to exposure of the blot to film. Protein bands were quantified by densitometry using ImageJ software version 1.41.
(National Institutes of Health, Bethesda, MD, USA), and normalised to β-actin as a loading control.

**Crystal violet cell number assay**

The number of macrophages present 24 h following LPS/IFNγ stimulation was measured using the crystal violet cell number assay as described previously (Yang et al., 1998). Briefly, at the 24 h time point the culture medium (including non-attached cells) was removed completely from cells grown in 96-well plastic culture plates. Attached cells were fixed by gentle shaking for 15 min in 1% glutaraldehyde in phosphate-buffered saline (100 µl/well) and plates rinsed 3 times by submersion in dH2O. Cells were air-dried overnight before staining with crystal violet (0.5% w/v in methanol, 100 µl/well) by gentle shaking for 20 min. Excess dye was removed by rinsing 4 times in dH2O and plates air dried for 1 h. The bound dye was solubilised in 10% acetic acid (100 µl/well) by gentle shaking for 15 min. The absorbance at 595 nm was measured using a microplate reader (Multiskan Ascent, Thermo Fisher Scientific, Milford, MA, USA). Cell number was calculated by comparison of sample absorbances with those of cell number standards (0–400,000 cells/well, seeded 4–6 h prior to fixing). A linear relationship between cell number and absorbance was observed over the range 80,000–400,000 cells/well.

**Tissue RNA extraction and real-time polymerase chain reaction (RT-PCR)**

Real-time PCR was used to measure IL-6, TNF-α, COX-2 and NOS-2 mRNA expression. Mouse lungs were pulverised in an RNase-free mortar and pestle under liquid nitrogen and homogenised by passing the tissue through a 21-gauge needle 5–10 times. Total RNA was isolated from a portion of the homogenised tissue using Qiagen RNeasy Mini kits, according to the manufacturer’s instructions. The extracted RNA was eluted into 50 µl RNase-free water.
and stored at –80°C until reverse transcription. RNA was diluted 1 in 10 in RNase-free water before 2.5 µl of this solution was reverse transcribed using Superscript VILO cDNA synthesis kits, according to the manufacturer’s instructions, in a final volume of 5 µl. The resulting cDNA was diluted with 195 µl ultra-pure water and stored at –20°C until RT-PCR analysis. RT-PCR was performed in triplicate for each gene of interest using a 384-well plate ABI Prism 7900HT sequence detection system (Applied Biosystems, Scoresby, VIC, Australia). Each 5 µl reaction consisted of 2 µl diluted cDNA, 2.5 µl Platinum SYBR Green qPCR Supermix-UDG, and 0.05 µM of the relevant forward and reverse primers (Table 1). Primer sequences were either obtained from published references, or designed using Primer Express software (Applied Biosystems) with mRNA sequences from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov, Bethesda, MD, USA). The threshold cycle value determined for each gene was normalised against that obtained for 18S ribosomal RNA, which was included as an internal control.

Data analyses

Results are expressed as mean ± standard error of the mean for n independent observations, where n represents the number of cell culture experiments conducted on different days, the number of individual blood donors, or the number of individual animals in each treatment group. All experiments were replicated at least three times. Some data are pooled from several independent studies, resulting in uneven group sizes. Where appropriate, data were statistically analysed using GraphPad Prism version 5.0b (GraphPad Software, La Jolla, CA, USA). In most cases, a one-way analysis of variance (ANOVA) was conducted and treatment groups were compared to the LPS+IFNγ control group (cell culture experiments) or LPS/vehicle control group (animal experiments) by Dunnett’s post-test. A P-value less than 0.05 was considered to be statistically significant.
Materials

2MEO, 17β-estradiol, dexamethasone, paclitaxel and ICI 182,780 were dissolved in 100% dimethylsulfoxide to a stock concentration of 10 mM and stored at −20°C until required. Lyophilised LPS and colchicine were dissolved in sterile dH₂O to stock concentrations of 1 mg·ml⁻¹ and 10 mM respectively and stored at −20°C. Lyophilised IFNγ was dissolved in 0.1% w/v bovine serum albumin in phosphate-buffered saline to a stock concentration of 1000 IU·ml⁻¹ and stored at −80°C. Primary antibodies for western blotting were diluted in 2% w/v bovine serum albumin with 0.1% w/v sodium azide in TBS-Tween; secondary antibodies were dissolved in 5% skim milk. The COX-2 rabbit polyclonal antibody and β-actin mouse monoclonal antibody were obtained from Abcam (Cambridge, UK); Lymphoprep from Axis-Shield (Oslo, Norway); mouse IL-6 and TNF-α OptEIA ELISA sets and human IL-6 and TNF-α matched antibody pairs from Becton Dickinson (North Ryde, NSW, Australia); protein assay kit and semi-dry transfer apparatus from Bio-Rad Laboratories (Gladesville, NSW, Australia); sheep anti-mouse IgG antibody and sheep anti-rabbit IgG antibody from Chemicon (Millipore, North Ryde, NSW, Australia); [³H]PGE₂, dextran T500, ECL reagent and nitrocellulose membranes from GE Healthcare (Rydalmere, NSW, Australia); GIBCO Dulbecco’s Modified Eagle’s Medium and RPMI 1640 Medium, Superscript VILO cDNA synthesis kits and all RT-PCR reagents from Invitrogen (Mulgrave, VIC, Australia); foetal calf serum from JRH Biosciences (Brooklyn, VIC, Australia); methoxyflurane from Medical Developments International (Springvale, VIC, Australia); sodium pentobarbital from Merial (Parramatta, NSW, Australia); ethidium bromide and acridine orange from Molecular Probes (Invitrogen, Mulgrave, VIC, Australia); scintillation fluid from PerkinElmer (Glen Waverley, VIC, Australia); RNeasy Mini kits from Qiagen (Doncaster, VIC, Australia); recombinant mouse interferon-γ from R&D Systems (Minneapolis, MN, USA); L-glutamine from SAFC
Biosciences (Sigma-Aldrich, Castle Hill, NSW, Australia); anti-PGE₂, bovine serum albumin, colchicine, crystal violet, dexamethasone, dextran, 17β-estradiol, HEPES, LPS (*Escherichia coli* serotype 0111:B4), non-essential amino acid solution, paclitaxel, penicillin/streptomycin solution, PGE₂, protease and phosphatase inhibitor cocktails, sodium bicarbonate and sodium pyruvate from Sigma-Aldrich (Castle Hill, NSW, Australia); 2MEO from Steraloids (Newport, RI, USA); plastic culture plates from NUNC (Thermo Fisher Scientific, Roskilde, Denmark); ICI 182,780 from Tocris (Ellisville, MO, USA); and the NOS-2 rabbit polyclonal antibody from Transduction Laboratories (Lexington, KY, USA).
Results

**Lipopolysaccharide and interferon-γ induce J774 macrophage activation**

In order to ascertain appropriate stimuli for use in this study, we examined the time course of NOS-2 and COX-2 protein expression and nitrite and PGE2 production as markers of J774 macrophage activation. Nitrite is a stable end product of the rapidly occurring oxidation of nitric oxide. Over a 24 hour period, LPS (1 µg·ml⁻¹) induced detectable expression of NOS-2 and COX-2 protein, but only a small increase in nitrite and PGE2 concentrations (Figure 1). In contrast, the combination of LPS and IFNγ (0.1 IU·ml⁻¹) elicited more rapid onset of protein expression and mediator production (detectable after 6 h), and over 24 h resulted in nitrite and PGE2 levels over 3-fold higher than those observed from cells stimulated with LPS alone. The combination of LPS and IFNγ was selected as the activating stimulus for use in subsequent experiments.

**2-Methoxyestradiol has no effect on J774 macrophage cell number**

As changes in cell number might confound the interpretation of measures of macrophage function, we examined the impact of LPS/IFNγ stimulation and 2MEO treatment on J774 cell number at the 24 h time point. 2MEO (10 µM) had not detectable effect on unstimulated cells, however stimulation with LPS/IFNγ significantly lowered cell number (Table 2). Treating LPS/IFNγ-stimulated cells with 2MEO (1–10 µM) caused no further reduction in cell number.

**2-Methoxyestradiol suppresses J774 macrophage activation to an extent comparable to that of dexamethasone**

When added 30 minutes prior to stimulation, 2MEO (0.1–10 µM) inhibited LPS/IFNγ-induced IL-6 and PGE2 production in a concentration-dependent manner over 24 h, by up to
30% and 47% respectively, at a concentration of 10 µM (Figure 2). 2MEO had no effect on LPS/IFNγ-induced TNF-α or nitrite production. These effects were compared with those of an established anti-inflammatory agent, the glucocorticoid dexamethasone at 0.1 µ M. This concentration of dexamethasone was greater than that required to saturate glucocorticoid receptors (Ballard and Ballard, 1972) and caused maximal inhibition of the response to LPS and IFNγ in J774 cells (data not shown). Dexamethasone markedly inhibited IL-6 (by 53%), TNF-α (by 61%) and PGE2 production (by 60%), but did not affect nitrite production. Neither 2MEO (10 µM) nor dexamethasone (0.1 µM) had any effect on inflammatory mediator production by unstimulated cells (Table 3). In line with its effects on nitrite and PGE2 production, 2MEO inhibited COX-2 protein expression (by 32% at 10 µM 2MEO) but had no effect on NOS-2 protein expression (Figure 2).

17β-Estradiol has no effect on J774 macrophage activation

Since 2MEO has low but readily detectable affinity for estrogen receptors (Hughes et al., 2002), and acts as an estrogen receptor agonist on estrogen receptor-positive breast tumour epithelial cells (Sutherland et al., 2005), we examined the role of estrogen receptors in the action of 2MEO on macrophage activation. 17β-estradiol has over 100-fold greater affinity for estrogen receptors than 2MEO and would be expected to saturate these receptors at concentrations of around 10–30 nM (Hughes et al., 2002). However, 17β-estradiol had no significant effect on LPS/IFNγ-induced IL-6, TNF-α, PGE2 or nitrite production at concentrations up to 1000 times greater than these (0.1–10 µM) (Figure 2).
The estrogen receptor antagonist ICI 182,780 does not prevent the effect of 2-methoxyestradiol

The estrogen receptor antagonist ICI 182,780 (K\textsubscript{d} ~0.4 nM for both α and β receptor subtypes) was used to further investigate whether high affinity estrogen receptors are involved in the action of 2MEO on J774 macrophages (Escande et al., 2006). ICI 182,780 (1 µM, a concentration sufficient to saturate estrogen receptors and outcompete binding of the lower-affinity ligand 2MEO) did not affect LPS/IFN\textsubscript{γ}-induced inflammatory mediator production in the absence of other treatments, nor did it prevent the suppression of LPS/IFN\textsubscript{γ}-induced IL-6 and PGE\textsubscript{2} production by 2MEO (Table 4). Interestingly, in the presence of ICI 182,780, the suppression of IL-6 production by 2MEO appeared to be modestly enhanced, and 2MEO now inhibited LPS/IFN\textsubscript{γ}-induced TNF-α production (by 27%).

The actions of 2-methoxyestradiol are not mimicked by the microtubule-interfering agents colchicine and paclitaxel

Given that microtubules are a known molecular target of 2MEO, we examined the effects of two microtubule-interfering agents on J774 macrophage activation. Colchicine inhibits microtubule polymerisation by binding to free tubulin, while paclitaxel stabilises microtubules by binding to the β-subunit of tubulin thereby preventing microtubule disassembly (Jordan et al., 1998). In contrast to the actions of 2MEO, both colchicine and paclitaxel (0.1–10 µM) inhibited LPS/IFN\textsubscript{γ}-induced nitrite production (by up to 15% and 19% respectively) while enhancing LPS/IFN\textsubscript{γ}-induced PGE\textsubscript{2} production (by up to 159% and 60% respectively) (Figure 3). Colchicine (but not paclitaxel) inhibited LPS/IFN\textsubscript{γ}-induced IL-6 and TNF-α production by up to 34% and 40% respectively. In addition, colchicine (10 µM) increased PGE\textsubscript{2} and nitrite production (by 78% and 14% respectively) in cells not stimulated with LPS/IFN\textsubscript{γ} (Table 3).
2-Methoxyestradiol suppresses activated human monocyte TNF-α production to an extent comparable to that of dexamethasone

In order to verify that the actions of 2MEO on J774 macrophages are not species or cell line dependent, we examined the effect of 2MEO on activated human peripheral blood monocytes. 2MEO (0.3–10 µM) inhibited LPS/IFNγ-induced TNF-α production by up to 60% at a concentration of 3 µM (Figure 4). In cells from the same donors, dexamethasone (0.1 µM) inhibited TNF-α production by 55%. 2MEO had no effect on LPS/IFNγ-induced IL-6 or PGE2 production by human monocytes, whereas dexamethasone suppressed production of these mediators by 66% and 79%, respectively. Human monocytes did not produce detectable concentrations of nitrite.

The glucocorticoid receptor antagonist RU486 partially reverses the effects of dexamethasone but not 2-methoxyestradiol

In order to investigate whether macrophage inhibition by 2MEO could be mediated via glucocorticoid receptors, we pre-treated human monocytes with the glucocorticoid receptor antagonist RU486. RU486 (1 µM) partially reversed inhibition of LPS/IFNγ-induced TNF-α by 0.1 µM dexamethasone, but had no effect on the inhibition of monocyte TNF-α production by 3 µM 2MEO (Table 5).

2-Methoxyestradiol suppresses lipopolysaccharide-induced inflammation in a mouse model of acute lung injury

We used a mouse model of acute lung injury to examine whether the actions of 2MEO in the J774 cell line would translate to acute anti-inflammatory activity in an in vivo setting. Intranasal administration of LPS causes an influx of inflammatory cells into the lungs and an
increase in BAL protein that peaks after 24 hours (Szarka et al., 1997). The increase in BAL cell number is attributed mostly to neutrophil accumulation, with a slight increase in monocyte/macrophage numbers (Bozinovski et al., 2004). The increase in BAL protein is caused by the vascular leak and epithelial injury that is associated with acute inflammation. 2MEO significantly inhibited the LPS-induced increase in BAL protein by 54% at a dose of 150 mg·kg⁻¹, but had no effect at 50 mg·kg⁻¹ (Figure 5). The effect of 150 mg·kg⁻¹ 2MEO was comparable to that of 1 mg·kg⁻¹ dexamethasone. On the other hand, at neither dose did 2MEO influence LPS-induced increases in BAL cell number (Table 6). Similarly, dexamethasone had no effect on this endpoint. The effects of 2MEO and dexamethasone on LPS-induced gene expression in the whole lung were examined for comparison with the actions of these agents on J774 macrophage activation. At a dose of 150 mg·kg⁻¹, 2MEO inhibited LPS-induced IL-6 mRNA expression by 63% (Figure 5). Dexamethasone had a similar effect on IL-6 mRNA. At the time point examined, no significant changes were detected in TNF-α, COX-2 or NOS-2 mRNA expression following treatment with 2MEO or dexamethasone (Table 6).
Discussion

We report the novel observation that 2MEO regulates LPS/IFNγ-activated J774 macrophages and human blood monocytes. Suppression of macrophage activation by 2MEO may account for some of the anti-inflammatory effects of this compound that cannot be attributed to its anti-angiogenic or anti-proliferative activity. The partial protective effects of 2MEO in the mouse model of LPS-induced acute lung injury support this conclusion. Although the effects of 2MEO are modest, in the acute model of inflammation they were comparable in magnitude (but not potency) to those of dexamethasone, a widely used anti-inflammatory agent. Investigations of the potential molecular mechanisms by which 2MEO could influence macrophage function revealed that despite affinity for estrogen receptors, 2MEO does not suppress macrophage activation via these receptors. Moreover, the macrophage modulatory profile of 2MEO is distinct from that of other microtubule-interfering agents. Our findings establish that 2MEO possesses anti-inflammatory activity not attributable to its anti-proliferative or anti-angiogenic actions, at doses similar to those having anti-tumour activity (Sutherland et al., 2007).

We examined the effect of 2MEO on the production of IL-6, TNF-α, PGE2 and nitric oxide by activated J774 macrophages as these mediators are functional outcomes of macrophage activation known to have direct, immediate and protracted impacts on inflammation (Nathan, 2002). 2MEO partially suppressed LPS/IFNγ-induced PGE2 and IL-6 production. Although modest and observed at relatively high concentrations, the magnitude of these reductions was similar to that of reductions elicited by the benchmarking anti-inflammatory glucocorticoid, dexamethasone. In J774 macrophages, neither 2MEO nor dexamethasone completely suppressed the LPS/IFNγ-induced increase in inflammatory mediator production, although it
is possible that other markers of macrophage activation may have been more susceptible to glucocorticoid inhibition (Linden and Brattsand, 1994). Neither 2MEO nor dexamethasone suppressed nitrite production by J774 cells. Apart from the 100-fold difference in their potencies, the main difference in the profiles of the two agents was that dexamethasone suppressed TNF-α production, whereas 2MEO was ineffective against TNF-α production in the mouse cell line. However, 2MEO did inhibit TNF-α production by LPS/IFNγ-stimulated primary human blood monocytes, suggesting that its effects are not species-specific or unique to the J774 cell line. The discordance in regulation of PGE2, IL-6 and TNF-α by 2MEO in murine and human cells may be due to a transformed cell line-primary cell difference or a mouse-human species difference (reviewed by Mestas & Hughes (2004)).

Our findings are consistent with the earlier observation that 2MEO inhibits activation of guinea-pig peritoneal macrophages (Stewart, 1999). The other study examining the direct effects of 2MEO on macrophage-like cells found that 2 μM 2MEO reduced osteoclast cell number by over 95% (Maran et al., 2006). However, we did not detect significant changes in cell number following treatment with 2MEO, suggesting that the actions of 2MEO in these two contexts are unrelated. Furthermore, the actions of 2MEO on PGE2 and IL-6 production cannot be attributed to cytotoxicity.

The molecular mechanism underlying the direct effects of 2MEO on macrophages remains unknown. The reversal of the inhibitory effects of dexamethasone by the glucocorticoid receptor antagonist RU486 confirms the mechanism of action of dexamethasone in this study. However, the observation that RU486 does not affect macrophage inhibition by 2MEO and the differences in the patterns of cytokine modulation of 2MEO and dexamethasone make it unlikely that they are acting via a common target. Furthermore, the chemical structure of
2MEO lacks a hydroxyl group at carbon number 11, a feature of steroidal compounds that is known to be required for biological activity as a glucocorticoid (Axelrod, 1976).

Recently estrogen receptor-β agonists have attracted attention as potential anti-inflammatory agents (Koehler et al., 2005). Previous studies in LPS/IFNγ-stimulated J774 cells found in one case modest suppression of nitric oxide production by 17β-estradiol (Hayashi et al., 1998), and in another case enhancement (Sakazaki et al., 2005), but did not examine other inflammatory mediators. However, in the present study concentrations of 17β-estradiol far greater than those required to saturate high-affinity estrogen receptors had no effect on J774 macrophage activation. Furthermore, the estrogen receptor antagonist, ICI 182,780, did not prevent the effects of 2MEO. Thus, it is unlikely that 2MEO modulates macrophage function via high affinity estrogen receptors. The mechanism underlying the modest enhancement of some of the inhibitory effects of 2MEO in the presence of ICI 182,780 is unclear.

2-Methoxyestradiol is known to destabilise microtubules by binding to the colchicine binding site of tubulin (Kd ~20 µM) (D’Amato et al., 1994). Microtubule-interfering agents such as colchicine and paclitaxel are cytotoxic to proliferating cells (Mollinedo and Gajate, 2003) and modulate macrophage function (Mantovani, 1982). While paclitaxel has been reported to elicit macrophage activation similar to that induced by LPS (Manthey et al., 1994), colchicine is known to suppress LPS-induced inflammatory cytokine induction (Rao et al., 1997), as was observed in the present study. Although colchicine reduced LPS/IFNγ-induced IL-6 production, its suppression of LPS/IFNγ-induced TNF-α and nitrite production (and enhancement of nitrite production by unstimulated cells) was not mimicked by 2MEO. Moreover, the enhancement of PGE2 production by colchicine in both stimulated and unstimulated cells suggests a very different pattern of macrophage modulation to that caused
by 2MEO. Although these contrasting patterns of response do not exclude microtubules as a molecular target for the effects of 2MEO on macrophages, they are not readily explained by a microtubule-related mechanism.

While the molecular mechanism of the macrophage effects of 2MEO in vitro remains to be elucidated, our observations encouraged an investigation of 2MEO in vivo. Alveolar macrophages are thought to play a major role in initiation of the innate immune response to LPS in the mouse model of acute lung injury (Koay et al., 2002; Maus et al., 2002). Unlike previous studies that have examined the anti-inflammatory effects of 2MEO in vivo, the acute nature of the current study excludes a role for either angiogenesis or tissue remodelling in the anti-inflammatory effects. Although macrophages may not be the only target of 2MEO in this model, the suppression of LPS-induced increases in BAL protein by 2MEO supports the hypothesis that macrophage-targeted actions of this compound make an important contribution to its anti-inflammatory properties. 2MEO may also directly limit vessel leak by downregulation of the HIF-1α transcription factor, resulting in reduced expression of VEGF, a pro-angiogenic cytokine originally identified as ‘vascular permeability factor’ (Yan et al., 2006). This action of 2MEO could explain why it reduced BAL protein but not cell number. Dexamethasone also failed to significantly reduce BAL cell number in the present study. However this finding is consistent with those reported by others in the mouse model of LPS-induced acute lung injury (Bozinovski et al., 2004), and is broadly consistent with neutrophilic inflammation showing less sensitivity to regulation by glucocorticoids.

The suppression of LPS-induced IL-6 gene expression that was observed in vivo is consistent with the changes in IL-6 production seen in vitro. One action of IL-6 is to increase permeability of pulmonary endothelial cells, thus contributing to increases in BAL protein (Fu
et al., 2009). However, gene expression measurements are based on whole lung RNA, and may reflect changes in the cellular composition of the lungs (such as LPS-induced neutrophilia or infiltrating monocytes) in addition to changed gene expression levels in resident cells such as alveolar macrophages.

In the LPS-induced lung injury model, drugs were administered 2 hours after challenge as this protocol is more likely to be clinically relevant in the context of acute inflammation. Both 2MEO and dexamethasone inhibited acute lung injury even when administered after the initiation of inflammation. Studies with 2MEO in other disease conditions have demonstrated that the low potency of this compound is related to its short half-life. We have shown that in BALB/c mice receiving 50 mg·kg⁻¹ 2MEO daily for 16 days, serum levels peak at 100–400 nM (Sutherland et al., 2005). However, doses as low as 15 mg·kg⁻¹ induced major estrogenic side effects when administered chronically. The in vivo doses of 2MEO used in the present study (50 or 150 mg·kg⁻¹) were selected to achieve a plasma concentration range consistent with that having regulatory effects on macrophages in vitro (1–10 µM); the dexamethasone dose (1 mg·kg⁻¹) was selected because this is a commonly used reference dose in comparative studies. Notwithstanding the reservations regarding the estrogenicity and low potency of 2MEO, its maximum effect was similar to that of dexamethasone, lending it therapeutic relevance. Pharmacological optimisation to generate more potent, selective and stable 2MEO analogues has the potential to generate a novel anti-inflammatory drug class (Hughes et al., 2002). Such optimisation needs to be guided, inter alia, by further elucidation of the mechanisms by which 2MEO modulates macrophage function (Sutherland et al., 2007).

In conclusion, our results demonstrate that 2MEO modulates monocyte and macrophage activation in vitro, and has acute anti-inflammatory activity in vivo. These findings provide an
explanation for anti-inflammatory actions of 2MEO that could not be accounted for by its well-known effects on cell proliferation and blood vessel development. The molecular mechanism by which 2MEO modulates macrophage function remains unclear, but it appears unlikely to act via estrogen receptors, glucocorticoid receptors or microtubules.
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Authorship Contributions

Participated in research design: Shand, Langenbach, Ziogas and Stewart.

Conducted experiments: Shand, Langenbach, Keenan, Ma, Wheaton, Schuliga and Stewart.

Performed data analysis: Shand, Keenan and Schuliga.

Wrote or contributed to the writing of the manuscript: Shand, Ziogas and Stewart.
References


Footnotes

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Figure 1. Time course of the response of J774 macrophages to LPS in the presence and absence of IFNγ. Cells were stimulated with LPS alone (1 µg·ml⁻¹), or LPS in combination with IFNγ (1 µg·ml⁻¹ and 0.1 IU·ml⁻¹, respectively). At each time point, cell supernatants were collected for measurement of (A) nitrite and (B) PGE₂ concentrations. Data are expressed as mean ± SEM (4 technical replicates, where error bars exceed the size of the symbols). Cell lysates were collected for western blot analysis of NOS-2 and COX-2 protein expression in (C) cells stimulated with LPS alone and (D) cells stimulated with LPS and IFNγ. β-Actin was used as a loading control.

Figure 2. Effect of 2MEO (■), 17β-estradiol (E2, ▲) and dexamethasone (DEX, shaded histogram) on inflammatory mediator production and COX-2 and NOS-2 protein expression by LPS/IFNγ-stimulated J774 macrophages. Cells were pre-treated with dexamethasone (0.1 µM, 2 h), 2MEO (0.1–10 µM, 30 min) or 17β-estradiol (0.1–10 µM, 30 min), before stimulation with LPS (1 µg·ml⁻¹) and IFNγ (0.1 IU·ml⁻¹). Twenty-four hours later, cell supernatants were collected for measurement of (A) IL-6, (B) TNF-α, (C) PGE₂ and (D) nitrite concentrations. Inflammatory mediator data are expressed as mean ± SEM (n = 5). The concentrations of IL-6, TNF-α, PGE₂ and nitrite for the LPS+IFNγ response were 43 ± 7 ng·ml⁻¹, 103 ± 11 ng·ml⁻¹, 14 ± 1 ng·ml⁻¹, and 57 ± 2 µM, respectively. In some experiments, cell lysates were collected eighteen hours after stimulation for western blot analysis of (E) COX-2 and (F) NOS-2 protein expression, relative to β-actin as a loading control (blots representative of results obtained in 3 independent experiments). ** P < 0.01 compared with LPS+IFNγ response (one-way ANOVA with repeated measures, Dunnett's
post-test); † P < 0.05, †† P < 0.01, ††† P < 0.001 compared with LPS+IFNγ response (paired t-tests).

**Figure 3.** Effect of colchicine (●) and paclitaxel (◊) on inflammatory signalling mediator production by LPS/IFNγ-stimulated J774 macrophages. Cells were pre-treated with colchicine or paclitaxel (0.1–10 µM, 30 min) before stimulation with LPS (1 µg·ml⁻¹) and IFNγ (0.1 IU·ml⁻¹). Twenty-four hours later, cell supernatants were collected for measurement of (A) IL-6, (B) TNF-α, (C) PGE₂ and (D) nitrite concentrations. Data are expressed as mean ± SEM (n = 5). The concentrations of IL-6, TNF-α, PGE₂ and nitrite for the LPS+IFNγ response were 43 ± 7 ng·ml⁻¹, 103 ± 11 ng·ml⁻¹, 14 ± 1 ng·ml⁻¹, and 57 ± 2 µM, respectively.

* P < 0.05, ** P < 0.01, *** P < 0.001 colchicine-treated group compared with LPS+IFNγ response; ^ P < 0.05, ^^^ P < 0.001 paclitaxel-treated group compared with LPS+IFNγ response (one-way ANOVA with repeated measures, Dunnett's post-test).

**Figure 4.** Effect of 2MEO and dexamethasone (DEX) on inflammatory signalling mediator production by LPS/IFNγ-stimulated human monocytes. Cells were pre-treated with dexamethasone (0.1 µM, 2h) or 2MEO (0.3–10 µM, 30 min), before stimulation with LPS (100 ng·ml⁻¹) and IFNγ (10 ng·ml⁻¹). Twenty-four hours later, cell supernatants were collected for measurement of (A) IL-6, (B) TNF-α and (C) PGE₂ concentrations. Data are expressed as mean ± SEM (n = 4). The concentrations of IL-6, TNF-α and PGE₂ for the LPS+IFNγ response were 5.5 ± 1 ng·ml⁻¹, 1.3 ± 0.3 ng·ml⁻¹, and 480 ± 130 pg·ml⁻¹, respectively.

* P < 0.05 compared with LPS+IFNγ response (one-way ANOVA with repeated measures, Dunnett's post-test); † P < 0.05 compared with LPS+IFNγ response (paired t-test).
Figure 5. Effect of 2MEO and dexamethasone (DEX) on LPS-induced increases in BAL protein in mice and on IL-6 gene expression in mouse lungs. Two hours after intranasal instillation of LPS (1 µg), mice received an intraperitoneal injection of 2MEO (50 or 150 mg·kg⁻¹) or dexamethasone (1 mg·kg⁻¹). Twenty-two hours later, mice were killed and, following BAL fluid collection for (A) protein measurement, their lungs removed. Lung RNA was extracted and mRNA expression for (B) IL-6 was determined by RT-PCR, relative to expression of the internal control 18S rRNA. Data are expressed as mean ± SEM (n = 9–14). * P < 0.05, ** P < 0.01 compared with LPS/vehicle response (one-way ANOVA, Dunnett's post-test).
Table 1. Primer sequences used for real-time PCR analysis.

<table>
<thead>
<tr>
<th>gene</th>
<th>forward primer (5′-3′)</th>
<th>reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>CCGGAGAGGGGAGCCTG</td>
<td>CTGCTGCCCTCCTTGAT</td>
</tr>
<tr>
<td>IL-6</td>
<td>TGTTCTTGGGAAAATCGTGA</td>
<td>TCAGAATTGCCATTCACAAC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CAGACCCCACACTCAGA</td>
<td>TGCTACGACGTGGGCTACAC</td>
</tr>
<tr>
<td>COX-2</td>
<td>GTCAGCCAGGCAGCAA</td>
<td>CCCACGTTTTCATGGA</td>
</tr>
<tr>
<td>NOS-2</td>
<td>ACAATGGCAATCAGTGGC</td>
<td>CCCTACTGCTGGG</td>
</tr>
</tbody>
</table>
Table 2. Effect of LPS/IFNγ stimulation and 2MEO treatment on J774 macrophage cell number. Cell number was measured by crystal violet assay 24 hours after stimulation and is expressed as mean ± SEM (n = 3). The cell number for the unstimulated control was $2.5 \pm 0.6 \times 10^5$ cells per well. * P < 0.05 compared with unstimulated control (one-way ANOVA with repeated measures, Bonferroni’s post-test).

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Treatment (30 min before stimulation)</th>
<th>Cell number (% unstimulated control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>Vehicle</td>
<td>100</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>2MEO 10 µM</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>LPS+IFNγ</td>
<td>Vehicle</td>
<td>75 ± 5 *</td>
</tr>
<tr>
<td>LPS+IFNγ</td>
<td>2MEO 1 µM</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>LPS+IFNγ</td>
<td>2MEO 3 µM</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>LPS+IFNγ</td>
<td>2MEO 10 µM</td>
<td>66 ± 6</td>
</tr>
</tbody>
</table>
Table 3. Effect of 2MEO, 17β-estradiol, dexamethasone, ICI 182,780, colchicine and paclitaxel on basal inflammatory mediator production by unstimulated J774 macrophages. Cell supernatants were collected 24–26 hours after treatment for measurement of mediator concentrations. Data are expressed as mean ± SEM ($n = 5$; $n = 4$ for dexamethasone and ICI 182,780 data). The concentrations of IL-6, TNF-α, PGE$_2$ and nitrite for the LPS+IFN-γ response were $43 ± 7$ ng·ml$^{-1}$, $103 ± 11$ ng·ml$^{-1}$, $14 ± 1$ ng·ml$^{-1}$, and $57 ± 2$ µM, respectively. * $P < 0.05$, ** $P < 0.01$ compared with vehicle response (paired $t$-tests).

<table>
<thead>
<tr>
<th>treatment</th>
<th>IL-6 (% LPS+IFN-γ response)</th>
<th>TNF-α (% LPS+IFN-γ response)</th>
<th>PGE$_2$ (% LPS+IFN-γ response)</th>
<th>nitrite (% LPS+IFN-γ response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>1.4 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>11.8 ± 4.4</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>2MEO 10 µM</td>
<td>1.5 ± 0.3</td>
<td>2.0 ± 0.4</td>
<td>15.2 ± 4.2</td>
<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>17β-estradiol 10 µM</td>
<td>1.5 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>6.7 ± 2.0</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>dexamethasone 0.1 µM</td>
<td>0.8 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>7.9 ± 3.1</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>ICI 182,780 1 µM</td>
<td>1.0 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>8.0 ± 1.0</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>colchicine 10 µM</td>
<td>1.1 ± 0.1</td>
<td>4.6 ± 1.0</td>
<td>78.0 ± 44.0 **</td>
<td>14.0 ± 3.7 *</td>
</tr>
<tr>
<td>paclitaxel 10 µM</td>
<td>3.2 ± 1.7</td>
<td>6.0 ± 3.8</td>
<td>31.9 ± 16.7</td>
<td>5.9 ± 0.9</td>
</tr>
</tbody>
</table>
Table 4. Effect of 2MEO on inflammatory mediator production by LPS/IFN\(\gamma\)-stimulated J774 macrophages in the presence and absence of ICI 182,780. Cells were pre-treated with ICI 182,780 (1 h) and/or 2MEO (30 min). Cell supernatants were collected 24 hours after stimulation for measurement of mediator concentrations. Data are expressed as mean ± SEM (\(n = 4–5\)). The concentrations of IL-6, TNF-\(\alpha\), PGE\(_2\) and nitrite for the LPS+IFN\(\gamma\) response were 43 ± 7 ng·ml\(^{-1}\), 103 ± 11 ng·ml\(^{-1}\), 14 ± 1 ng·ml\(^{-1}\), and 57 ± 2 \(\mu\)M, respectively. ** \(P < 0.01\) compared with LPS+IFN\(\gamma\) response; ^ \(P < 0.05\), ^^ \(P < 0.01\) compared with LPS+IFN\(\gamma\) response in the presence of ICI 182,780 (one-way ANOVA with repeated measures, Dunnett’s post-test).

<table>
<thead>
<tr>
<th>treatment (LPS/IFN(\gamma)-stimulated cells)</th>
<th>IL-6 (% LPS+IFN(\gamma) response)</th>
<th>TNF-(\alpha) (% LPS+IFN(\gamma) response)</th>
<th>PGE(_2) (% LPS+IFN(\gamma) response)</th>
<th>nitrite (% LPS+IFN(\gamma) response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ICI 182,780 1 (\mu)M</td>
<td>88 ± 5</td>
<td>89 ± 8</td>
<td>73 ± 6</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>2MEO 10 (\mu)M</td>
<td>70 ± 5 **</td>
<td>89 ± 9</td>
<td>53 ± 9 **</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>2MEO+ICI 182,780</td>
<td>55 ± 7 ^^</td>
<td>73 ± 6 ^</td>
<td>53 ± 5 ^</td>
<td>93 ± 6</td>
</tr>
</tbody>
</table>
Table 5. Effect of 2MEO and dexamethasone on TNF-α production by LPS/IFNγ-stimulated human monocytes in the presence and absence of RU486. Cells were pretreated with RU486 (1 μM, 2.5 h), dexamethasone (0.1 μM, 2h) and/or 2MEO (3 μM, 30 min). Cell supernatants were collected 24 hours after stimulation for measurement of TNF-α concentrations. Data, expressed as a percentage of the response in vehicle treated cells, are presented as mean ± SEM (n = 4). The concentration of TNF-α for the LPS+IFNγ response was 2.1 ± 0.9 ng·ml⁻¹. * P < 0.05 compared with LPS+IFNγ response; ^ P < 0.05 compared with LPS+IFNγ response in the presence of dexamethasone (paired t-tests).

<table>
<thead>
<tr>
<th>treatment (LPS/IFNγ-stimulated cells)</th>
<th>TNF-α (% LPS+IFNγ response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>100</td>
</tr>
<tr>
<td>RU486 1 μM</td>
<td>119 ± 14</td>
</tr>
<tr>
<td>2MEO 10 μM</td>
<td>76 ± 11 *</td>
</tr>
<tr>
<td>2MEO+RU486 0.1 μM</td>
<td>65 ± 14</td>
</tr>
<tr>
<td>dexamethasone 0.1 μM</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>dexamethasone+RU486</td>
<td>72 ± 10 ^</td>
</tr>
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</table>
Table 6. Effect of 2MEO and dexamethasone on LPS-induced increases in BAL cell number in mice and on TNF-α, COX-2 and NOS-2 gene expression in mouse lungs. Mice were killed 24 h after LPS instillation for BAL fluid collection and removal of the lungs. Lung mRNA expression was determined by real-time PCR, relative to expression of the internal control 18S rRNA. Data are expressed as mean ± SEM (n = 9–14).

<table>
<thead>
<tr>
<th>treatment (administered 2 h after LPS instillation)</th>
<th>BAL cell number (×10^6)</th>
<th>TNF-α mRNA (relative to saline/vehicle control)</th>
<th>COX-2 mRNA (relative to saline/vehicle control)</th>
<th>NOS-2 mRNA (relative to saline/vehicle control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline/vehicle</td>
<td>0.32 ± 0.02</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>LPS/vehicle</td>
<td>1.80 ± 0.16</td>
<td>3.3 ± 0.5</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>LPS/2MEO 50 mg·kg⁻¹</td>
<td>1.61 ± 0.18</td>
<td>3.4 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>LPS/2MEO 150 mg·kg⁻¹</td>
<td>1.74 ± 0.26</td>
<td>2.7 ± 0.6</td>
<td>1.3 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>LPS/dexamethasone 1 mg·kg⁻¹</td>
<td>1.48 ± 0.19</td>
<td>2.0 ± 0.4</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 1

Graph A: Nitrite (µM) vs. time after stimulation (h)
- Control
- LPS
- LPS + IFNγ

Graph B: PGE₂ (ng·mL⁻¹) vs. time after stimulation (h)
- Control
- LPS
- LPS + IFNγ

Graph C: LPS stimulus
- NOS-2
- COX-2
- β-actin

Graph D: LPS + IFNγ stimulus
- NOS-2
- COX-2
- β-actin
Figure 2

A. IL-6 (% LPS+IFNγ response)

B. TNF-α (% LPS+IFNγ response)

C. PGE₂ (% LPS+IFNγ response)

D. Nitrite (% LPS+IFNγ response)

E. Western blot for COX-2 and β-actin

F. Western blot for NOS-2 and β-actin
Figure 3

**A**

IL-6

(% LPS+IFNγ response)

![Graph showing IL-6 response](image)

**B**

TNF-α

(% LPS+IFNγ response)

![Graph showing TNF-α response](image)

**C**

PGE2

(% LPS+IFNγ response)

![Graph showing PGE2 response](image)

**D**

Nitrite

(% LPS+IFNγ response)

![Graph showing nitrite response](image)
Figure 4

A

IL-6
(% LPS+IFNγ response)

B

TNF-α
(% LPS+IFNγ response)

C

PGE2
(% LPS+IFNγ response)
Figure 5

A

BAL protein (µg)

350
300
250
200
150

saline/vehicle
vehicle
2MEO 50 mg kg⁻¹
2MEO 159 mg kg⁻¹
DEX 1 mg kg⁻¹
LPS

B

IL-6 mRNA

(relative to saline/vehicle control)

50
40
30
20
10
0

saline/vehicle
vehicle
2MEO 50 mg kg⁻¹
2MEO 159 mg kg⁻¹
DEX 1 mg kg⁻¹
LPS